

IMMUNOMODULATION BY SHIGA TOXIN 2

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By

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Abstract

The Shiga-like toxins have DNA sequence homology to the toxins accountable for the dysentery brought about by the *Shigella* species. *Escherichia coli* which encode and produce shiga-like toxins are referred to as shiga toxin-producing *E. coli* (STEC). Upon infection with STEC, humans may develop a variety of clinical symptoms ranging in severity from bloody diarrhea to life threatening hemolytic uremic syndrome (HUS). Hemolytic uremic syndrome is the most fatal disease manifestation for humans and has been documented to occur in up to 20% of patients upon STEC infection [29]. The Shiga toxins (Shiga toxin 1 and 2) are regarded as the principal virulence factor of STEC and are responsible for the clinical manifestations during HUS in humans [49].

Cattle are the primary non-human reservoir for STEC and therefore represent an attractive target for pre-slaughter intervention as a means to reduce human infections. To date, vaccination with secreted proteins including Shiga toxin 2 (Stx2), has reduced the numbers of bacteria shed in feces [3]. Even though published data exists supporting vaccination in cattle as a means to reduce STEC, commercially available vaccines are not being used by farms and STEC remain a significant zoonotic pathogen of humans causing disease and death. To further our knowledge about STEC pathogenesis in cattle, we examined the effect of Shiga toxin 2 on bovine immune responses. Bovine lymphocyte function was determined in the presence of Shiga toxin 2 and the magnitude of bovine immunological responses was measure after immunization with Shiga toxin 2. In general, results suggest that Shiga toxin 2 downregulates bovine immune responses suggesting vaccination with effector molecules that exclude Shiga toxin 2 may induce a better immunological response and improve vaccine efficacy.

To examine the possibility that Stx2 modulates bovine immune responses, we investigated lymphocyte function in the presence of Stx2. Menge et al [70] have reported that bovine lymphocytes express the Stx receptor and that Shiga toxin 1 inhibits lymphocyte proliferation *in vitro*. We isolated two populations of lymphocytes, peripheral blood mononuclear cells (PBMCs) and ileal Peyer's patch lymphocytes (IPPL) and compared lymphocyte function in the presence and absence of Stx2. We found that Stx2 did not affect

IPPL viability *in vitro* but did inhibit IPPL proliferation after 12 hours of incubation *in vitro*. In contrast, no altered PBMC function could be observed in the presence of Stx2. These results suggest that receptor-bound Stx2 may inhibit IPPL proliferation and that the two populations of lymphocytes isolated are unique and distinct from each other in their response to Stx2.

To determine the effect of Stx2 on bovine immune responses during STEC infection, a bovine ileal ligated loop model was employed. Ligated loops were inoculated with either a Stx2⁺ STEC strain or an isogenic Stx2⁻ STEC strain. After 24 hours, IPPL populations were isolated from each ligated loop and immunophenotyped. The results indicated a significantly reduced CD4⁺ T cell population in the presence of Stx2. No differences in the levels of IFN α , TNF α , IL12 or IFN γ could be detected between groups. These results suggest that Stx2 modulates bovine immune responses but not as a result of increased production of these cytokines. To extend this finding, we determined the effect of Stx2 on bovine immune responses during active immunization by using ELISA to measure serological responses in the presence and absence of Stx2. Serological responses to secreted proteins, as well as a co-administered antigen (hen egg lysozyme), were significantly reduced in the groups of cattle that were immunized with either purified Stx2 or secreted protein preparations isolated from STEC compared to groups vaccinated with antigens which did not contain the toxin. Bovine proliferative responses were also measured and the results indicated significantly reduced proliferation in the groups vaccinated with the formulations containing Stx2. Therefore, based on these results, we conclude that Stx2 downregulates bovine immune responses and thus may contribute to the colonization and persistence of cattle by STEC.

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Dedication

To Michael, whose patience and confidence
have been unwavering.

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List of Abbreviations

A/E	Attaching and effacing
CO ₂	Carbon dioxide
CDT	Cytolethal distending toxin
DNA	Deoxyribonucleic acid
ddH ₂ O	Double-distilled water
<i>E. coli</i>	<i>Escherichia coli</i>
EHEC	Enterhaemorrhagic <i>E. coli</i>
EDTA	Ethylenediaminetetrmcetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
GALT	Gut-associated lymphoid tissue
Gb ₃	glycosphingolipids possessing terminal gal α 1-4gal disaccharides
GIT	Gastrointestinal tract
HC	Haemorrhagic colitis
HUS	Haemolytic uremic syndrome

IFN	Interferon
IL	Interleukin
IPP	Ileal Peyer's Patch
JPP	Jejunal Peyer's patch
LPS	Lipopolysaccharide
MALT	Mucosal-associated lymphoid tissue
MAP	Mitogen activated protein
MDa	Mega dalton
MEM	Minimal essential medium
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NC	Needle complex
NF- κ B	Nuclear factor- κ B
OD	Optical density
OM	Outer membrane
Orf	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg/ml	Picograms/milliliter
qRT-PCR	Quantitative real-time polymerase chain reaction

RNA	Ribonucleic Acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STEC	Shiga-toxin producing <i>E. coli</i>
Stx	Shiga toxin
TLR	Toll-like receptor
Tir	Translocated intimin receptor
T3SS	Type III secretion system
Tween 20	Polyethylenesorbitan monolaurate 20
TGN	Trans-golgi network
μl	Microliter
USA	United States of America
UV	Ultra violet
WASP	Wiskott-aldrich syndrome protein

1.0 Literature Review

Escherichia coli

1.1.1. Nomenclature

E. coli normally colonizes the human gut within hours after birth and participates in maintenance and stability of gastrointestinal physiology. Some *E. coli* strains have evolved the ability to cause human diseases through the acquisition of specific virulence factors. Pathogenic *E. coli* are divided into four main groups i) diarrheagenic *E. coli*, ii) uropathogenic *E. coli*, iii) meningitis *E. coli*, and iv) avian pathogenic *E. coli* [1]. Diarrheagenic *E. coli* can be further divided into six pathotypes including one of the most important human pathogens, Enterohaemorrhagic *E. coli* (EHEC).

1.1.2. STEC O157:H7

Enterohaemorrhagic *E. coli* are a subgroup of Shiga toxin-producing *E. coli* (STEC). Shiga toxin-producing *E. coli* includes all strains of *E. coli* that produce at least one shiga toxin. After much debate, scientists have agreed to formally define EHEC as “those STEC which have been demonstrated to cause diarrhea in humans” [2]. This includes the EHEC prototype serotype *E. coli* O157:H7. Thus, by this definition, not all STEC strains are pathogenic to humans whereas all EHEC strains do cause disease [3]. The identification of EHEC as a defined group of pathogenic *E. coli* was a consequence of two unrelated findings. First, Riley et al [4] showed that *E. coli* O157:H7 was the causative agent of haemorrhagic colitis (HC) during two outbreaks involving undercooked hamburger meat. Second, in 1983 Karmali et al [5] discovered a cytotoxin in the stool of patients experiencing haemolytic uremic syndrome (HUS) and subsequently, it was shown that this was the same toxin that was being produced by the *E. coli* O157:H7 during the two 1982 outbreaks. This cytotoxin was later identified as shiga toxin. Enterohaemorrhagic *E. coli* has been further divided based on the presence of virulence attributes into “typical EHEC” which includes strains that produce stxs, cause A/E lesions and possess the 60-MDa plasmid. These strains include *E. coli* O157:H7 and “atypical EHEC” which produce shiga toxins but do not produce A/E lesions and/or the 60-MDa plasmid [3]. The absence of these virulence factors does not indicate decreased virulence in humans; in fact Paton et al [6]

showed that an atypical EHEC strain lacking the ability to produce A/E lesions was frequently associated with HC and HUS.

The most common means of characterizing STEC strains is through serotyping. The serotype of an *E. coli* isolate is based on its O and H antigens. The O antigen is part of the cell wall lipopolysaccharide. 174 O antigens had been described although less than 10 O groups are responsible for the majority of human disease [7]. The H antigen is the flagella subunit, 53 H antigens had been described [6]. The most relevant serotype is *E. coli* O157:H7 as it predominates in many countries and human infection has been described on at least 6 continents [3]. *E. coli* O157:H7 is also most commonly associated with HUS [1]. The number of documented human infections of *E. coli* O157:H7 within the United States has increased between the years 1982 and 1998 [1]. In 2005, human infections with *E. coli* O157:H7 had increased to 2,621 cases within the United States [1]. Other countries experiencing large outbreaks of *E. coli* O157:H7 infections include Canada, Japan and the United Kingdom [1]. *E. coli* O157:H7 can be identified through a number of biochemical tests including a delayed D-sorbitol fermentation rate, the inability to ferment rhamnose on agar plates and the inability to produce β -glucuronidase. The completed genome of *E. coli* O157:H7 showed that it had acquired an additional 1.34-Mb region of genomic DNA not present in the genome sequence of non-pathogenic *E. coli* K-12 [8].

1.1.3. Non-O157 Serotypes

Recent data has confirmed that many non-O157 serotypes are responsible for human infection. Regions such as Australia, Latin America and Europe have experienced sporadic outbreaks due to O26, O103 and O145 serotypes [9]. A global assessment done by Hussein et al [10] in 2007 showed that up to 30% of ground beef was contaminated with non-O157 STEC and up to 50% contamination of other retail cuts. To date, the majority of non-O157 serotypes associated with human disease are O26:H11, O103:H2, O111:NM (non-motile) and O113:H21 [3]. In North America, epidemiological data suggests that approximately 20% of HUS cases are due to non-O157:H7 serotypes [3]. However, many non-O157 serotypes have been shown to cause less bloody diarrhea and less HUS overall and are often routinely isolated from stool samples.

1.1.4. Human Diseases

Most of the time, infection with *E. coli* O157:H7 is self-limiting, but severe renal and neurological complications develop in approximately 5% of cases [11].

1.1.4.1. Diarrhea and Hemorrhagic colitis

The most common clinical manifestations in humans after ingestion of EHEC are diarrhea, HC and HUS [3]. The initial complaint is usually a nonbloody diarrhea with abdominal cramps and a short-lived fever. The onset of nonbloody diarrhea ranges between 2 and 12 days but most often occurs between 3 and 4 days [9]. Vomiting occurs in approximately half of the cases usually around the same time as the nonbloody diarrhea. Within a couple of days, 90% of patients experience bloody diarrhea with increased abdominal pain.

1.1.4.2. Hemolytic uremic syndrome

Hemolytic uremic syndrome is the most often fatal disease manifestation for humans following STEC infection and has been documented to occur in up to 20% of STEC infected patients [12]. It has been suggested that the Stxs produced by STEC traverse the intestinal epithelial layer to the bloodstream. Once in the bloodstream, Shiga toxins target tissues with high levels of the Stx receptor. In humans, renal endothelial cells have a high Gb₃ content and it has been shown that Stxs are cytotoxic to these cells *in vitro*. Various cytokines such as TNF α and IL1 β increase expression of Gb₃, exacerbating the cytotoxic effects of the Stxs [9]. Patients with HUS often exhibit elevated levels of these cytokines in serum and urine [32].

It is estimated that HUS occurs at a frequency of 2.1 cases per 100,000 persons (within the total population) [12]. Acute renal failure is seen in 55 to 70% of cases with patients experiencing HUS [9]. The Center for Disease Control estimated that there are approximately 70,000 cases of STEC induced illness including 60 deaths per year within the United States [13]. The occurrence of HUS is seasonal with a higher frequency in the summer months. The progression to HUS is aided by a variety of factors including age (the very young and the elderly), patients experiencing bloody diarrhea and fever, and patients treated with antimotility agents [9]. Recurring cases of HUS are not common. In one study in Utah, HUS occurred twice in the same patient only 2.6% of the time over a 20 year span [9]. Approximately 10% of

children under the age of ten experiencing illness due to infection with STEC develop HUS. Without proper treatment, 3-5% will die due to complications associated with HUS [9].

1.1.5. Animal Reservoirs

Bacterial zoonotic pathogens are a threat to public health around the world. Ruminants are an important source of STEC and are considered to be the primary means of transmission to humans leading to infection and disease.

1.1.5.1. Bovine

Shiga toxin-producing *E. coli* is the only group of pathogenic *E. coli* that have a zoonotic origin as either direct or indirect animal contact has been shown to be a principal transmission route [3]. Cattle are the most important source of STEC causing human disease as the majority of outbreaks have been associated with undercooked ground beef or water contaminated with bovine feces [3]. As well, cattle are asymptomatic excretors of STEC and shed STEC in their feces all year round [14]. Shiga toxin-producing *E. coli* can remain viable in faeces from several weeks to many months facilitating spread [15]. It has been documented that fecal shedding is higher during the warmer summer months of the year and is influenced by the age of the animal as younger calves excrete higher amounts of STEC than older cattle [15]. Therefore since cattle are asymptomatic reservoirs, this makes detection of STEC difficult because infected animals are not removed from the herd due to illness or death. In 2003, Naylor et al [16] proposed that the terminal rectum was the predominant site of *E. coli* O157:H7 colonisation within the bovine host although conflicting data suggested the bovine fore-stomachs. Later Nart et al [17] confirmed Naylor's findings and in addition also identified minor sites of *E. coli* O157:H7 colonisation within the rumen, small intestine and the proximal colon.

1.1.5.2. Non-bovine Species

Other reservoirs for *E. coli* O157:H7 include sheep, water buffalo, rabbits and horses [7]. Both meat and milk from sheep are relevant sources of STEC and goat milk has been linked to an STEC outbreak [18]. Over 100 STEC serotypes have been isolated from sheep [7]. Buffalo dairy herds in southern Italy were shown to be colonized with STEC and thus another potential

source for infection. *E. coli* O157:H7 has also been isolated from pigs in Europe, Japan and the United States. Faecal carriage was reported to be low (2%) and due to possible contamination of foodstuff or poor husbandry practices [15]. Both *E. coli* O157:H7 and non-O157 serotypes have been isolated from gull droppings suggesting a potential vector for the organisms. Also, STEC have been isolated from pigeons and turkeys [15]. Finally, STEC has been isolated from bird droppings which may implicate another potential vehicle in STEC spreading [15].

1.1.6. Modes of transmission

Shiga toxin-producing *E. coli* has a number of vectors aiding its transmission to humans. The fact that STEC is able to survive in water, feces, animal feed and on inanimate barnyard surfaces aids in its transmission.

1.1.6.1. Food and Water

Meat, milk and water have been the primary sources of STEC infection [15]. Undercooked beef was a source of STEC infection in North America between the months of December 1992 and January 1993. The undercooked beef was sold at a fast food chain and resulted in the infection of 732 individuals within the states of Washington, Idaho, California and Nevada. Out of the 732, 195 persons were hospitalized and 4 died in the outbreak. In May 2000, the Walkerton water supply was contaminated with numerous pathogenic organisms including *Salmonella*, *Campylobacter* and *E. coli* O157:H7. Both adults and children were affected by this contamination which resulted in approximately 2300 cases of gastrointestinal illness with 28 cases of HUS verified. As a result, one child died of HUS and eight required kidney dialysis for recovery [19].

There are an increasing number of STEC infections arising from fruits and vegetables indirectly associated with contamination with cattle feces [4]. Fruits and vegetables fertilized with ruminant manure or contaminated during harvesting have been implicated as potential sources of STEC [15]. For example, bean sprouts, lettuce and tomatoes have been associated with STEC outbreaks [15]. A multistate outbreak of *E. coli* O157:H7 infections occurred in the United States in the summer of 1997 and epidemiological studies revealed the causative agent to be alfalfa sprouts grown from seeds contaminated with *E. coli* O157:H7 originating from a farm

in Idaho [20]. An outbreak in 2006 in the United States revealed spinach had been contaminated with *E. coli* O157:H7 as a result of coming into contact with contaminated water [21]. Studies revealed that the water had been contaminated with bovine feces [15]. Shiga toxin-producing *E. coli* has been shown to be quite resilient as it can remain viable on lettuce at 4°C for 15 days. Finally, unpasteurized milk and fruit juices, such as apple juice, have become a safety concern [22, 23]. A study done by Cody et al [22] showed that apples contaminated with *E. coli* O157 were grown in orchards that were frequented by deer that tested positive for *E. coli* O157:H7.

1.1.6.2. Environmental Exposure

Various environment-related events have been associated with STEC exposure. Examples include swimming in contaminated water or attending open air events such as agriculture fairs resulting in exposure to contaminated animals [15, 24].

1.2 STEC Virulence factors

Since *E. coli* O157:H7 is an extracellular pathogen, it is important for it to successfully colonize its host before being cleared by the immune system. Much effort has been put into elucidating the mechanism by which *E. coli* O157:H7 successfully colonizes its hosts. The two main virulence factors that STEC possesses are a pathogenicity island (PAI) encoding a type III secretion system as well as to expression one or more Stxs.

1.2.1. Type III Secretion system

It is known that *E. coli* O157:H7 intimately attaches to the lymphoid follicle-dense mucosa in the terminal rectum resulting in attaching-effacing (A/E) lesions [25]. The genes required for A/E lesions are encoded on a PAI termed LEE (locus of enterocyte effacement). The locus of enterocyte effacement encodes a type III secretion system (TTSS) consisting of approximately 40 open reading frames within five operons. The first four operons are designated *LEE1*, *LEE2*, *LEE3*, and *LEE4*. *LEE1-3* contains the genes required for the structural components of the TTSS that spans the bacterial inner and outer membranes [26]. *LEE4* encodes proteins necessary for filament and pore formation. These include EscF, EspA, D, F and B. The fifth operon encodes proteins having effector activity within the host cell including Tir, intimin

and the Tir chaperone, Ces T [27]. One of the control mechanisms regulating LEE expression is the LER protein. The LER activates transcription of the LEE genes by binding to the upstream regulatory region of LEE2 [28].

Type III secretion systems are exclusive to gram negative bacteria and are required for directly transporting bacterial proteins (referred to as effector proteins) from the bacterial cytosol into host cells [27]. This requires the formation of a needle complex that is able to span the inner bacterial membrane, peptidoglycan layer, the outer bacterial membrane and as well across the host cellular membrane and into the host cell interior [29]. In *E. coli* O157:H7, the surface translocon structure is a key component of the needle complex. The translocon is made by the polymerization of EscF and the long filament portion is composed of EspA [29]. EspA subunits are assembled in a helical structure which develop into a cylindrical structure with a central channel [30]. The extent of the translocon structure is determined genetically [31]. Physical contact between EspA and EspB has been confirmed. EspB (along with EspD) forms pores of approximately 3-5nm in diameter in the host cell membrane [32]. EspB is also translocated into the host cytosol and is involved in signal transduction events that provoke effacement of microvilli and the formation of pedestal-like structures, a prerequisite for the intimate attachment of *E. coli* O157:H7 to host cells [31]. Physical contact between EspB and EspD has been demonstrated [31]. EspD acts as a capping protein, as well as an anchor to connect the filament to the epithelial cell [1]. CesD, has been shown to be a secretion chaperone for the EspD protein and is needed for the overall extracellular secretion of EspB [33].

The “export” apparatus of the needle complex is mainly located in the inner membrane of the bacterial cell and is required for targeting and propelling secretion of the outer membrane proteins as well as the effector proteins. The export apparatus is composed of EscD, EscR, EscU, EscV, EscS and EscT proteins and is also associated with the cytoplasmic ATPase EscN [26]. The ring-shaped outer membrane protein in *E. coli* O157:H7 is EscC and is in direct contact with the translocon needle that protrudes from the bacterial cell [26]. The inner and outer membrane protein structures are connected by EscJ [26].

One of the most important T3SS effector proteins of *E. coli* O157:H7 is Tir (translocated intimin receptor). Tir is a multi-functional protein serving as a receptor for the bacterial outer membrane protein intimin and is also involved in activating host signalling pathways leading to

actin rearrangement [25]. Once inserted into the host cellular membrane, Tir adopts a hairpin-like topology revealing an extracellular domain that binds to intimin, leaving the amino- and carboxy-terminal regions intracellular [34]. In eukaryotic cells, actin polymerization is under the control of the Arp2/3 complex. This complex is stimulated by the WASP (Wiskott-Aldrich syndrome protein) family of proteins, particularly the N-WASP protein [25]. Subsequently, N-WASP is activated by the Rho-family GTPases, such as Nck [25]. Therefore, *E. coli* O157:H7 recruit N-WASP to sites of infection through Tir in order to cause actin rearrangement and pedestal formation within host cells. Recently it has been shown that residues 454-463 within *E. coli* O157:H7 Tir are crucial for pedestal formation [25]. The discovery of another bacterial protein called Tccp encoded by EHEC, was also shown to induce pedestals by the activation of N-WASP [35]. Tir also redirects the recruitment of host cytoskeletal proteins such as α -actinin, ezrin, coractin, talin, fimbrin, vasodilator-stimulated phosphoprotein, and actin-related protein complex in order to provoke pedestal formation [36].

Finally, the binding of intimin to its receptor Tir, initiates the intimate attachment of bacteria to intestinal epithelial cells increasing bacterial numbers and prolonging fecal shedding [31]. To date, intimin is the only agreed upon *E. coli* O157:H7 adherence factor shown to participate in intestinal colonization *in vivo* in an animal model [3]. Various subtypes of intimin have been shown to exist. Enterohaemorrhagic *E. coli* O157:H7 strains expressing intimin- α produce A/E lesions in both the small and large intestines whereas EHEC strains producing intimin- γ produce A/E lesions in the large intestine in gnotobiotic piglets [31]. The different subtypes of intimin therefore influence tissue tropism in different hosts.

1.2.2. Fimbrial Adhesins

E. coli O157:H7 contains a highly conserved plasmid called pO157 that was proposed by Karch et al [37] to encode fimbrial adhesions that allowed bacteria to attach to Henle407 epithelial cells. Since this study, conflicting data has emerged and the role of pO157 in adherence is controversial. Hall et al [3] reported that loss of the plasmid had no effect on adhesion in serotype O5:H- whereas other serotypes did show reduced adhesion. Oral inoculation of adult rabbits with strains that did not possess the plasmid showed decreased adherence to intestinal cells but the presence or absence of the plasmid made no difference in the

amount of diarrhea observed. A critical limitation in establishing the role of pO157 is the lack of suitable animal models that replicate all aspects of the human disease. After culturing *E. coli* O157:H7, Maneval et al [37] described rigid fimbrial structures on the surface of the bacteria but after sequence analysis, pO157 revealed no fimbrial gene cluster. Despite the conflicting data about pO157, epidemiological data has shown that it is extensively distributed among human EHEC isolates. A study done by Levine et al [3] revealed that 99% of 107 *E. coli* O157:H7 human isolates possessed the plasmid.

1.2.3 Nonfimbrial Adhesins

Many *E. coli* O157:H7 nonfimbrial adhesins have been suggested to play a role in bacterial pathogenesis. Paton et al [38, 39] showed that antibodies to LPS blocked bacterial adhesion to Henle407 cells whereas the pre-treatment of the same cells with LPS did not block *E. coli* O157:H7 adherence. This suggested that the antibodies were sterically hindering the LPS structure and thus physically blocking adherence [1]. Another nonfimbrial adhesin, a 94-kDa outer membrane protein associated with adherence to Henle407 cells and which is immunologically distinct from intimin [37]. Tarr et al [9] reported that an outer membrane protein termed Iha, located on O-islands 43 and 48 possibly involved in *E. coli* O157:H7 adherence. Also, the identification of a large outer membrane protein called ToxB encoded on pO157 has been implicated in *E. coli* O157:H7 adherence. ToxB has homology to toxins A and B from *Clostridium difficile*. ToxB facilitates the secretion of TTSS hence playing a role in full bacterial adherence. Enterohaemorrhagic *E. coli* cured of their pO157 plasmid resulted in lower numbers of bacterial colonies on Caco-2 cells and reduced secretion of EspA, EspB and Tir [7]. The Efa1 (EHEC factor for adherence) has homology to ToxB and was detected when an *efal* mutant showed decreased adherence to hamster ovary cells [7]. Further studies showed that Efa1 contributes to bovine intestinal colonization. Another nonfimbrial adhesin important for bovine colonization is Saa which has been shown to be associated with STEC isolated from cattle rather than from humans [7]. Finally, the outer membrane protein A (OmpA) seems to be an *E. coli* O157:H7 nonfimbrial adherence factor besides being associated with many other functions such as porin activity and conjugation mediation [1].

1.2.4. Secreted proteases

Shiga toxin-producing *E. coli* encode several proteases that are suggested to contribute to the progression of disease in humans. These proteases are not involved in the production of A/E lesions and often their functions are unknown. *E. coli* O157:H7 secretes a plasmid-encoded serine protease termed EspP. EspP has sequence homology to EspC of EPEC and both are known as autotransporters mediating their own secretion through the bacterial outer membrane [37]. One of the substrates of EspP is human coagulation factor V which suggests that *E. coli* O157:H7 may interfere with the human blood clotting cascade. Not all *E. coli* O157:H7 that encode EspP produce EspP as shown in one study where only three of five *E. coli* O157:H7 isolates produced EspP as detected by Western blots [40]. EspI is a serine protease functionally related to EspP. EspI degrades pepsin A and again human coagulation factor V [7]. Shiga toxin-producing *Escherichia coli* strains also secrete EpeA which is another plasmid-encoded serine protease which has mucinase activity.

1.2.5. Hemolysin

STEC produce a plasmid-encoded hemolysin termed Ehly 1-associated protein. Ehly 1-associated protein was first characterized by Beutin et al [41] by the production of small turbid zones of haemolysis on blood agar plates. This description suggested a defective mechanism of secretion resulting in only small incomplete zones of haemolysis although clones of serogroup O103 have been isolated that produce large clear zones of haemolysis suggesting an overproduction of Ehly 1-associated protein [37]. Ehly 1-associated protein is a member of the repeats in toxin (RTX) family of toxins and has been shown to be expressed by a range of other pathogens [41]. It is hypothesized that Ehly contributes to disease in humans through the lysis of red blood cells and the subsequent release of haemoglobin as a source of iron. Chart et al [37] showed that production of Ehly 1-associated protein can be increased by growing cells under conditions of low oxygen tension and anaerobic conditions which are similar to the environment found in the gut of humans and animals.

Ehly has been recently used as an epidemiological marker to detect STEC strains since many common serogroups such as O157, O26 and O111 produce Ehly 1-associated protein [37]. A plasmid-based probe CVD419 showed good correlation with Ehly 1-associated protein

production and was subsequently used to confirm the presence of the *ehly* gene. In 1992 a study done by Barrett et al [42] showed that 28 of 28 *E. coli* O157:H7 isolates were CVD419 probe-positive. Also in 1992, 29 of 48 non-O157 STEC isolates were found to be CVD419 probe-positive and 125 of 224 cattle isolates were CVD419 probe-positive [42]. Surprisingly, when the same 224 cattle were tested for the *eae* gene, only approximately 62 were positive [37]. Schmidt and Karch found the incidence of Ehly 1-associated protein was higher in STEC isolates causing HUS compared to STEC isolates causing only diarrhea suggesting its importance in the development of HUS [43].

1.2.6. Other possible virulence factors

Other important virulence factors encoded by many STEC strains include a 3169 amino acid protein termed cytolethal distending toxin (CDT), that has similarities to the large clostridial toxins (LCT) A and B [37]. Large clostridial toxins possess an N-terminal glucosyl-transferase that disrupts cell architecture suggesting that CDT encoded by STEC strains also acts through a similar mechanism. Recent studies have also shown that CDT is lethal for two lines of human vascular endothelial cells suggesting it may be involved in intestinal damage [7].

A very high percentage of *E. coli* O157:H7 strains possess the enteroaggregative heat-stable toxin (EAST1) that was first identified in the pathotype Enteroaggregative *E. coli* (EAggEC). The role of the toxin in human infection remains unknown but it has been shown to induce fluid secretions in a rabbit ileal model suggesting it may induce watery diarrhea in humans [37].

Sequence analysis of pO157 has revealed a potential catalase/peroxidase encoded by the gene *katP*. The exact role of the enzyme has yet to be determined but other bacterial catalases have been shown to neutralize cytotoxic oxidants produced by host macrophages thus aiding in bacterial survival [37]. To date the *katP* gene has not been found in sorbitol-fermenting *E. coli* O157:H7 and its distribution among O157 isolates is inconsistent.

The flagella produced by STEC can play an important role in pathogenesis. A study done by Erdem et al [44] showed that H7 and H6 flagellin have adhesive properties that bind mucins I and II, and contribute to the colonization of cattle. These results were later verified using a H7 *fliC* mutant which showed reduced ability to adhere to a bovine terminal rectal epithelial cell

line, but adherence was restored through complementation [45]. However, complementation analysis with the heterologous *fliC* from H6 was unable to restore H7 adherence properties.

Finally, recent studies by Klapproth et al [37] have shown that some O157 isolates may be capable of inhibiting lymphocyte activation. No specific gene(s) have been identified to attribute to this phenotype, but research on EPEC has shown that the gene(s) are non-LEE encoded. Further studies are required to determine their significance and possible role in EHEC virulence.

1.3 Toxins

Toxins possess very specific biochemical activities enabling them to either stimulate or disturb various cellular processes [46]. The different properties, targets and mechanisms of toxins are all important considerations when studying toxin biology [46]. Many bacterial toxins interfere with fundamental host functions therefore causing cellular death [47]. While other pathogens that are able to persistently colonize their host often use toxins to avoid the immune response by modulating a variety of cellular functions. Often, bacterial toxins are not toxic at biological concentrations required to achieve persistent colonization.

Currently, researchers have found ways to use toxins to advance science and the medical field. For example, tetanus and cholera toxins have been used to decipher critical steps in cellular pathways such as in signal transduction and regulated secretion [47]. The botulism toxin, one of the most toxic poisons known to humans is now used cosmetically and medically to treat wrinkles and urologic disorders [48]. This is made possible due to the extreme potency and specificity of the toxin which allows for the dose of the toxin to be so small that it remains at a biological concentration that is not toxic to its host.

1.3.1 Shiga toxin family

In 1898, a Japanese scientist by the name of Kiyoshi Shiga discovered “Shiga toxin” during an epidemic of dysentery in Japan due to *Shigella dysenteriae* [49]. We now define the Shiga toxin family as a group of genetically and functionally related molecules consisting of two groups (Shiga toxin from *S. dysenteriae* and Stx1 from *E. coli* form group 1 and the Shiga toxin

2 family forms group 2) with five members including Shiga toxin 1, Shiga toxin 2, Shiga toxin 2c, Shiga toxin 2d and Shiga toxin 2e [49].

Shiga toxin 1 is a homogenous family of toxins and is neutralized by anti-serum to Shiga toxin [14]. Shiga toxin 1 variants exist that differ by one amino acid but toxicity remains unaltered [14]. Shiga toxin 2 is very heterogeneous possessing variants of human and animal origin and is therefore divided into a number of subtypes [4]. Two variants, one isolated from a pig and the other from a patient with HUS, have been isolated that are immunologically related to Stx2 [4]. DNA sequencing of the two isolates revealed that the genome of the porcine strain does not possess a toxin-converting bacteriophage [50]. This Stx2 variant is termed Stx2e and is associated with edema disease in pigs, produced by strains E57, S1191 and 412 and is only weakly active on HeLa cells *in vitro* [51]. The other Stx2 variant isolated from the HUS patient was shown to be produced by strain H.I.8 and is more closely related to Stx2e [14]. In 2000, 20 variants of Stx2 had been reported with varying degrees of homology to Stx2 and Shiga toxin from *S. dysenteriae* I [52]. Shiga toxin 2 and Stx2c have been repeatedly isolated from humans with HUS and Stx2d is not usually associated with cases of diarrhea [15]. Finally, Stx2f has been isolated from STEC with an avian origin [15].

1.3.1.1. History and Nomenclature

The Shiga toxins are regarded as the principal virulence factor of STEC. The history of the Shiga toxins produced by *Enterobacteriaceae* was first described by Conradi in 1903 [7, 51]. Then in 1977, Konowalchuk described an *E. coli* cytotoxin that was lethal for Vero (African green monkey kidney) cells and was distinct from other *E. coli* toxins such as *E. coli* heat-stable toxin or heat-labile toxin [51]. Later in 1983, O'Brien et al [51] showed that the cytotoxin was indistinguishable in physiochemical features and biological activity to the Shiga toxin produced by *Shigella dysenteriae* I and was therefore named Shiga-like toxin (SLT). Shiga toxin (or SLT) produced by *E. coli* O157:H7 was demonstrated to be the causative virulence factor for HC and HUS in humans [51]. DNA sequencing of the *E. coli* O157:H7 genome in 2001 revealed that the *E. coli* O157:H7 genome had 1,387 genes that were not found in the non-pathogenic K12 genome [8]. Many of these genes were located on pathogenicity islands of bacteriophage origin including Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). Later, British researchers found that

stx1 and *stx2* gene expression was coordinated with prophage gene induction and for this reason alone, antibiotics are not recommended upon infection with STEC as they increase the amounts of free Stx in the gut lumen [8]. To date, 18 prophage and prophage-like elements have been recognized in the EDL933 genome [8]. Two of these include CP933V (Shiga toxin 1 encoding prophage) and BP933W (Shiga toxin 2 encoding prophage) [8].

1.3.2. Shiga toxin 2

The clinical significance of Stx2 in humans compared to Stx1 is linked to a more serious course of disease and is more important in the development of HUS. For example, an STEC outbreak occurred in Japan where 131 patients were infected with a Stx1-producing STEC serotype. In this outbreak, no cases of HUS were documented and the gastrointestinal symptoms were comparatively mild [53]. To further this point, a study on sporadic HUS cases in humans was completed. The results of this particular study revealed that 94% of HUS cases involved STEC capable of producing Stx2 alone or a combination of Stx2 and Stx1 [54]. Finally, animal studies have demonstrated that Stx2 is more fatal than Stx1 in mice [55] and gastrointestinal pathology is worse in rabbits infected with Stx2⁺ strains of STEC than Stx1⁺ strains [56].

1.3.2.1. Biological Activity

The Shiga toxins are comprised of 1 A subunit and 5 B subunits. The A subunits of Stx1 and Stx2 share approximately 57% nucleotide sequence homology and the B subunits share approximately 60% homology [51]. The A subunit has a molecular weight of approximately 32 kDa and the B subunit approximately 7 kDa [57]. The B subunits of Stx2 are identical but the A subunits differ which leads to variations in lethality. The B subunit is responsible for recognizing and binding to the blood-group-active glycolipid receptor globotriaosyl ceramide (Gb₃) which has the structure Gal α [1-4]Gal β [1-4]Glc-ceramide [39]. On the other hand, the Stx2e variant uses globotetraosyl ceramide, Gb₄ [58]. Only two toxins are known to use glycolipid receptors; the cholera toxin and the shiga toxins. All Stxs linked to human disease recognize the Gb₃ receptor. Upon receptor recognition and binding, the A subunit of the toxin is internalized via receptor-mediated endocytosis followed by retro-grade transport to the Golgi apparatus, the endoplasmic reticulum (ER) and finally translocation into the cytoplasm. The A

subunit is proteolytically cut between Ala253 and Ser254 resulting in a 28-kDa N-terminal peptide with enzymatic activity (A1 peptide) that is linked by a disulfide bond to a 4-kDa peptide (A2 peptide) [14]. The N-glycosidase activity of the A subunit targets the 28S rRNA where it cleaves a specific N-glycosidic bond thereby removing a single adenine residue subsequently preventing elongation-factor-1-dependent binding of aminoacyl-tRNAs to ribosomes which inhibits protein elongation thus killing the cell [57].

1.3.2.2. Structure

Structural analysis of Stx2 by Jackson et al [59] showed that a hydrophilic region at the N-terminus of the B subunit was responsible for the binding activity of Stx2 and another region at the C-terminus was required for extracellular localization. Crystal and NMR structure analyses of the B subunit have revealed it is comprised of two three-stranded anti-parallel β -sheets and an α -helix [52].

Crystal structures of Stx1 and Stx2 have revealed four major differences in their structures [60]. First, the active site of the A subunit was found to be much more accessible in Stx2 when compared to Stx1. Second, the presence of an α helix in the carboxy terminus of the A1 peptide of Stx2 allows for binding at a receptor-binding site whereas this site remains unoccupied in the Stx1 structure. Third, one of the carbohydrate binding sites in the B subunit has a different conformation when compared to Stx1 and this conformational variability may lead to different binding affinities for the Gb₃ receptor. Finally, the carboxy terminus of the A1 peptide of Stx2 binds Gb₃ in contrast to the A1 peptide of Stx1 [38].

13.3 Genetics of Shiga toxins

As early as the 1920s, scientists began to discover the role of bacteriophages in bacterial pathogenicity. Frobisher and Brown were the first to record that nontoxigenic streptococci acquired the ability to produce scarlatinal toxin after exposure to filtered supernatants of toxigenic streptococci [61]. Many examples exist of bacteriophage encoded toxin genes within bacteria including the pertussis toxin in *B. avium*, the botulinum toxin in *C. botulinum*, the diphtheria toxin in *C. diphtheria* and the Shiga toxins (Stx1 and Stx2) in *E. coli* [62].

1.3.3.1. Bacteriophage Origin

Shiga toxin 1 is encoded by a gene on a λ -like prophage called CP-933V and the gene for Shiga toxin 2 is encoded on a λ -like prophage called BP-933W [8]. Both Stx1 and Stx2 production is controlled by the bacteriophage lifecycle. When a lambdoid phage lysogenizes a cell, the specific phage repressor inhibits transcription of most phage genes and the prophage is in a quiescent state. Removal of the repressor results in the induction of the lytic cycle and the transcription of phage-encoded genes such as *stx1* and *stx2*. The *stx* genes are located in λ -like prophages within the late gene operon suggesting that they are co-ordinately expressed following prophage induction from the late phage promoter (p_R') [63]. Wagner et al [63] showed in 2001 that *stx2* gene expression relies primarily on the p_R' promoter and therefore on prophage induction from the lysogenic state to the lytic state. They constructed a mutant prophage that was deleted for the p_R' promoter region and results showed a significant reduction in Stx2 protein production.

1.3.4. Shiga toxin Receptor

Glycolipids are ubiquitous membrane components that display cell type-specific patterns and are present in bacteria, plant and animal species. Both bacteria and plants express glycosylglycerolipids but animals express glycosphingolipids (GSLs) [64]. There are four classes of glycosphingolipids based on their carbohydrate core structure and these include the gangliosphingolipids, the globosphingolipids, the lactosphingolipids and the mucoglycosylceramides [65]. All Shiga toxins specifically recognize and bind to the globo series GSLs such as Gb₃ [58]. Apart from being the Shiga toxin receptor, Gb₃ is also the Pk antigen of the P blood group system and Gb₃ is also known as CD77 which is the Burkitt's lymphoma antigen [66].

In 1998, Menge et al [67] demonstrated that bovine lymphocytes including peripheral blood mononuclear cells (PBMCs) and intraepithelial lymphocytes (IEL) express surface glycolipids, specifically the Gb₃ receptor. Subsequently they showed the immunomodulatory effects of purified Stx1 on bovine PBMCs was through the inhibition of mitogen induced proliferation [68]. Later the same group of researchers concluded that Stx1 directly affects bovine lymphocytes suggesting that the immune system is a major target for Stxs in cattle [69].

Cattle are the primary nonhuman reservoir for STEC and the pathogenicity of STEC for cattle is low. Some experimentally infected calves developed bloody diarrhea but no disease state in adult cattle has been associated with STEC infection [70]. Some researchers have hypothesized that the lack of disease in cattle has contributed to its spread to humans and therefore much effort has been put into understanding how STEC is able to persist asymptotically in the bovine host.

1.3.4.1. Structure

Glycolipids are composed of one or multiple monosaccharides glycosidically bonded to a lipid moiety [64]. In general, an amide bond links the fatty acid moiety to the amino group of the sphingosine which forms the ceramide portion of the molecule. As just mentioned, the ceramide portion is also glycosidically bonded to a carbohydrate head. Often the lipid moiety is hidden in the cellular membrane bilayer extruding the carbohydrate moiety on the cell surface. The structure of Gb₃ is shown in Figure 1.

1.3.4.2. Interaction of Gb₃ with Shiga toxins

Initial Stx-cell interaction experiments were done using cultured cell lines which revealed that only sensitive cells were able to remove Stx bioactivity from the overlying culture media [71]. Further experiments demonstrated that the addition of tunicamycin, an inhibitor of N-linked glycosylation, inhibited Stx activity indicating that Stx binds to a carbohydrate-containing receptor [71]. Upon the advent of purified iodinated Stx, direct binding studies were possible and in 1983 Brown et al [72] using thin-layer chromatography, proposed that glycosphingolipids were the Stx receptor. They showed that Stx bound to glycosphingolipids possessing terminal gal α 1-4gal disaccharides such as Gb₃ [71]. Also, Jacewicz et al [73] showed that Stxs bind to globotriosylceramide isolated from rabbit jejunum and a few years later, Lindberg et al [71] confirmed the requirement for the terminal gal α 1-4gal disaccharide for Stx binding. As well, Mobassaleh et al [74] demonstrated that rabbit jejunal membranes showed age-dependent susceptibility to fluid secretions caused by Stxs which correlated with age-dependent Gb₃ levels. Finally, loss of eukaryotic cell susceptibility to Stxs was shown through a variety of experiments including the inhibition of Stx-binding using monoclonal antibodies which bound to Gb₃,

destruction of Gb₃ by digestion of membrane glycolipids, and through the inhibition of Gb₃ biosynthetic pathways [75].

Cellular susceptibility to Stxs is not determined exclusively by Gb₃ content. It was demonstrated that the ceramide (lipid) portion of Gb₃ had a direct role on Shiga toxin recognition and binding as Shiga toxin did not bind to a lipid-free oligosaccharide nor to an oligosaccharide bound to bovine serum albumin [76]. Subsequently Lingwood et al [58] showed that the ceramide portion of Gb₃ determined the orientation and portion of the carbohydrate moiety displayed on the cellular surface thus affecting the interaction of the oligosaccharide head group with Stx. They found that unsaturated fatty acids with chain lengths between 20 and 22 showed the highest binding affinity for Shiga toxin 1 and chain lengths of 18 for Shiga toxin 2.

It is understood that carbohydrates have a very poor affinity for monovalent binding therefore multivalent interactions of ligands and receptors are necessary for efficient binding [64]. In 1998, Hooper et al [77] showed that membrane GSLs are bunched together in the outer leaflet of the membrane bilayer which can contribute to both the structural rigidity of the outer leaflet and efficient multivalent ligand interactions. This finding agreed with the X-ray crystallography structures generated of Shiga toxins bound to the Gb₃ receptor analogue Pk-MCO which revealed three trisaccharide molecules bound to each B subunit monomer of Stx. Therefore this finding implies that each B subunit has three distinct sites postulated to interact with Gb₃ and that there are 15 bound trisaccharides per Stx pentamer [64].

1.3.4.3. Receptor Distribution

The distribution of the glycolipid receptor amongst various eukaryotic cell types greatly influences the pathogenesis of disease. In humans, high levels of Gb₃ are found in the cortical region of the kidney which is also the primary site of renal lesions in HUS patients [38]. Studies using human, baboon and mouse kidneys showed similar amounts of Gb₃ in their cortex and medulla regions and Stxs were primarily associated with the tubular epithelial cells [38]. Takeda et al [78] also showed increased levels of tubular injury markers during the early stages of HUS which coincided with the number of HUS patients displaying tubular necrosis.

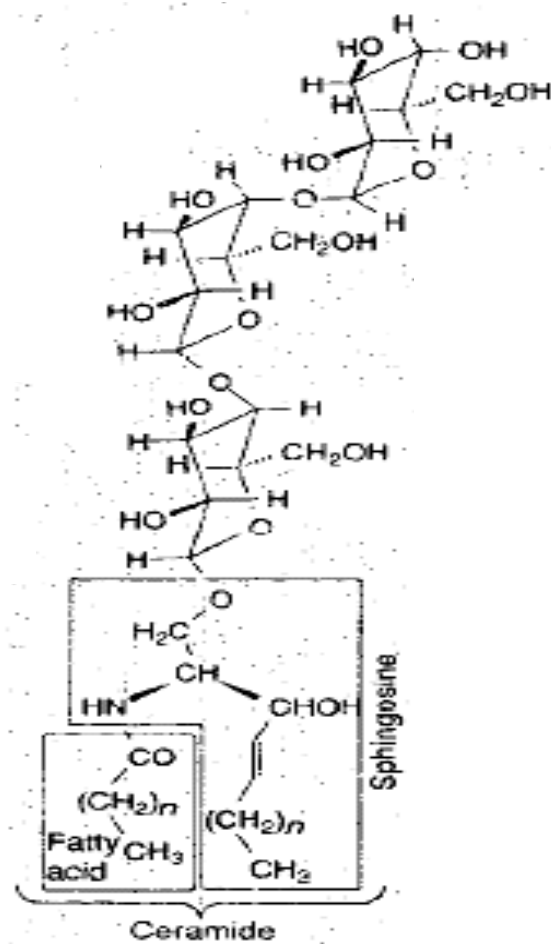


Figure 1. Structure of Gb₃ as presented by Lingwood et al [58].

The finding that red blood cells possess the glycolipid receptor on their surface may also influence the pathophysiology of HUS. As previously mentioned, the human P blood group antigens are glycolipids. More specifically, the Pk antigen is Gb₃, the P antigen is Gb₄ and the P1 antigen is a neolactoceramide containing a Gal α 1-4Gal moiety [38]. In 1990, Taylor et al [38, 79] hypothesized that red blood cells may remove Stxs from the circulation and in turn protect Gb₃-possessing tissues. Subsequently, *in vitro* studies showed that purified Stxs are able to bind to red blood cells but direct red blood cell-Stx binding during human STEC infection has yet to be investigated [38].

The Shiga toxins are able to bind to human endothelial cells and majority of histopathological lesions related to HUS are due to this interaction [38]. Early *in vitro* analyses revealed the susceptibility of cultured endothelial cells to the Stxs through loss of viability. The addition of certain cytokines such as TNF α and interleukin-1 β enhanced Stx cytotoxicity and this was shown to be a result of the upregulation of Gb₃ on the cell surface [38]. However, experiments using cultured endothelial cells have been difficult to interpret because cells isolated from different human tissues exhibit varying properties. For example, human renal and intestinal microvascular endothelial cells do not display increased sensitivity after pre-incubation with either TNF α or lipopolysaccharide (LPS) whereas human cerebral endothelial cells do [38].

1.3.5. Entry of Shiga toxin 2

After binding to the receptor Gb₃, Stxs are exclusively internalized through clathrin-coated pits [80]. Shiga toxin internalization can be inhibited by the removal of clathrin pits using hypotonic shock followed by potassium depletion as well as when the formation of the pit vesicle is inhibited by acidification of the cytosol [80]. After endocytosis, internalized Stx can follow several pathways. One route used by Stx-resistant cells involves the rapid transport of Stxs to lysosomes where they are degraded. A second possible route is through retro-grade transport of endosomal Stxs to the cytosol via the Endoplasmic Reticulum (ER) and the Golgi apparatus is required for intoxication. It has been suggested that Stxs first enter the Golgi apparatus from endosomes using a pathway dependent on a GTP-binding protein called Rab9. A third possible route might include the transport of endosomal Stxs directly to the trans-Golgi network (TGN) via the endosomal perinuclear recycling compartment [80]. From the TGN, it

has been reported that Stxs are transported to the ER by a Rab6-dependent process and that Stxs finally reach the cytosol by the Sec61 translocator complex [81]. Johannes et al [82, 83] showed that the B subunit of Stx rather than holotoxin is sufficient to guide retro-grade transport to the ER but that the B subunit does not possess a trafficking sequence in the carboxy-terminus end that many proteins that traffic to the ER via the Golgi do. Subsequently, it was demonstrated that the lipid portion of Gb₃ rather than Stx itself contains the trafficking sequence necessary to drive the retro-grade transport of Stxs to the cytosol [75]. Thus, treatment of cells with butyric acid has been shown to sensitize cells by changing their lipid composition (increasing fatty acid chain length) and therefore increasing Stx transport to the Golgi apparatus [81].

1.3.6. Mode of Action of Shiga toxin

The A subunit of Shiga toxin contains a loop formed by an internal disulfide bond. This loop is very sensitive to trypsin and cleavage results in the formation of A₁ and A₂ subunits. The A₁ subunit is enzymatically active and is necessary for inhibiting protein synthesis in cells [81]. Studies using site-directed mutagenesis altering the trypsin-sensitive site revealed that Stx retained cytotoxicity for cultured epithelial cells but that the effect was grossly delayed [81]. This pointed to the possibility of an alternative processing pathway most likely dependent on cellular enzymes. Two enzymes were subsequently demonstrated to cleave the A subunit of Stx strategically located in different areas of the cell. Garred et al [75] first showed that furin, a calcium-sensitive serine protease, was responsible for cleaving the A subunit within endosomes and the TGN. Furin recognizes the consensus motif Arg-X-X-Arg, which is found in the loop region of the A subunit while cleavage occurs optimally at low pH [75]. Also the cytosolic protease calpain was shown to cleave Stx as inhibition of calpain prevented cellular toxicity.

It is well accepted that the enzymatically active A₁ subunit must enter the cytosol in order to contact ribosomes and inhibit protein synthesis [81]. The A₁ subunit has been shown to be responsible for cleaving an adenine base from the 28S rRNA at position 4324 thereby inhibiting peptide chain elongation by preventing aminoacyl-tRNA binding to the acceptor site on the ribosome. [75]. Therefore the holotoxin undergoes transport to the Golgi apparatus and along the way the A₁ subunit may be generated by furin in the endosome and/or TGN and released

from the A₂ subunit in the ER [75]. If for some reason holotoxin or whole A subunit reaches the cytosol, calpain provides a less effective means for proteolytic cleavage [75].

1.3.7. Role of Shiga toxins in Pathogenesis

Since STEC are exclusively non-invasive pathogens, limiting their colonization to the intestinal mucosa, the translocation of Stxs from the gut lumen to underlying tissues and the bloodstream is assumed since infection with STEC causes systemic sequelae in both humans and animals. However, Stxs have not been detected in the blood of patients with HUS [52]. Clinical manifestations of patients experiencing HUS show that the gastrointestinal tract and kidneys are the organs most commonly affected and the vascular endothelial cells of these organs are the primary targets. Thrombotic lesions have also been reported in HUS patients in the microvasculature of the pancreas and brain [38]. Based on both *in vivo* and *in vitro* studies, the Shiga toxins have been shown to have multiple potent effects which include i) cytotoxicity ii) enterotoxicity and iii) paralytic activity. These effects have been shown to be dependent upon the targeted organ and specific cell type [84].

1.3.7.1. Gastrointestinal Tract

It has been hypothesized that Shiga toxins breach the intestinal epithelium either through lesions in the mucosal barrier and/or the destruction of tight junctions providing the portal of egress for Stxs into the circulation. In 2002, Hoey et al [85] showed that the bovine intestinal epithelium including the ileum and jejunum express the Gb₃ receptor. The direct translocation of toxins through epithelial cells has also been shown using polarized cell lines. Damage of the intestinal epithelium was shown using a ligated rabbit ileal loop model where purified Stx was inoculated directly into the loops [86]. Direct enterotoxic properties including mucosal inflammation and fluid accumulation, resulted in altered glucose and amino acid absorption. O'Loughlin et al [75] have hypothesized that this increased mucosal inflammation enhances luminal Stx absorption increasing the amount of intact Stx into systemic circulation. How the Stxs cross the epithelial barrier, studies have shown that the vascular endothelial cells are the primary target of Stxs within the GI tract. The diarrhea induced by infection with STEC in humans is due at least in part to exposure of enterocytes to Stx in the gut. Purified Stx was

shown to induce haemorrhagic lesions on the large intestine and thrombosis in the blood vessels of the mesentery in rabbits [75]. Tashiro et al [87] showed that the Stxs produce hemorrhagic lesions in the small intestine in rats. As well Sjogren et al [75] used a rabbit model to show the presence of microvascular thrombosis in the submucosal vessels of the cecum and colon when infected with Stx-producing STEC compared to those infected with Stx-negative strains. In contrast, studies using piglets showed that the presence of Stx did not affect the outcome of disease [88].

1.3.7.2. Kidneys

Studies using cultured cell lines revealed that Stxs are cytotoxic for renal endothelial, epithelial and mesangial cells [75]. The human kidney expresses high levels of the Stx receptor, Gb₃ in both the cortex and medulla which is consistent with the histopathological differences observed in patients diagnosed with HUS [75]. The principal histopathological changes associated with HUS include swollen and detached glomerular endothelial cells and deposition of fibrin in the renal microvasculature [38]. This led to the belief that vascular endothelial cells in certain organs, including the kidneys, are specifically targeted by Stxs. Lingwood et al [58] showed that fluorescein-conjugated Stx binds to renal distal convoluted tubules, collecting ducts, glomeruli and proximal tubules and another study done by Uchida et al [75] showed that Stx binds to both renal endothelial and epithelial cells in distal tubules. Also, β_2 microglobulin levels are elevated in HUS patients indicating considerable tubular dysfunction [75]. No small animal model of HUS is currently available although studies using rabbits and mice have resulted in noteworthy conclusions. Studies using mice fed *E. coli* K12 containing either the *stx1* or *stx2* gene cloned into a high-copy plasmid revealed acute renal bilateral cortical tubular necrosis and after 5 days, all of the mice that were exposed to Stx2 died. Also, rabbits exposed intraperitoneally to STEC developed acute focal tubular necrosis in the kidneys as well as diarrhea and cecal lesions [75]. Similar results were observed when the rabbits were exposed only to purified Stx2 [89]. These studies led to the hypothesis that Stx2 is more commonly associated with HUS than Stx1. Epidemiological studies have since revealed that Stx2-producing STEC are more often associated with outbreaks of HUS than Stx1-producing STEC [52]. In mice, Stx2 has been found to be 400-fold more potent than Stx1 and when Stx2 and LPS

were administered together, there was enhanced lethality [55]. Also, passive immunization of mice with anti-Stx2 has been shown to be protective as these mice do not develop renal failure contrary to the mice that received anti-Stx1 antibodies after STEC infection [75]. One possible explanation to account for these findings is that the *in vivo* level of transcription of *stx2* is higher since *stx1* is iron repressible. An alternative explanation is that the *stx2* gene is a clonal marker for STEC strains possessing additional virulence factors [38].

1.4 The Immune System

The cells of the immune system can be divided into two groups based on function. The first group are antigen non-specific and include monocytes/macrophages, natural killer (NK) cells, platelets and polymorphonuclear granulocytes. The polymorphonuclear granulocytes are further divided into neutrophils, eosinophils and basophils [90]. These cells are part of the innate immune response that is mounted early in an infection due to their constitutive expression and relative non-specificity [91]. Thus, innate immunity provides the time required for the antigen-specific cells or the adaptive immune response to develop. The second group of cells are the antigen-specific cells or the lymphocytes. These are part of the adaptive immune response which is inducible, highly specific and expresses positive memory [92].

1.4.1 Innate Immunity

The innate immune system is a front-line barrier eliciting an immune response between 0-4 hours after exposure to a pathogen. Within the gastrointestinal tract, the epithelial cells physically join together (a phenomenon known as tight junctions) to serve as a physical barrier and a first line of defense against pathogens. The apical surface of the epithelial cells is coated in a continuous layer of mucus and glycocalyx which is produced by goblet cells [93]. The innate immune system has three effector mechanisms including i) the phagocytic cells (including neutrophils, macrophages, natural killer cells and dendritic cells) ii) the complement system and iii) antimicrobial peptides including defensins and cathelicidins.

1.4.1.1. Phagocytic Cells

Phagocytosis is the uptake of foreign material by neutrophils, macrophages and dendritic cells. STEC uses its functional type III apparatus to limit its uptake by preventing the F-actin polymerization required for uptake [94]. The ability of STEC to control this uptake by phagocytes is due to effectors EspF and EspJ, which are responsible for the inhibition of FcγR- and CR3 mediated phagocytosis [95]. Phagocytic cells are activated by the presence of invading pathogens through innate immune signalling receptors such as Toll-like receptors (TLRs) which are present on the surface of antigen presenting cells such as macrophages and dendritic cells. Toll-like receptors are of germline origin and they have evolved to recognize molecules not typically found in mammalian cells and are hence called pathogen-associated molecular patterns (PAMPs) [96]. Toll-like receptors contain three major domains. First, the amino-terminal domain consists of a series of leucine rich repeats (LRRs) followed by a transmembrane domain and a C-terminal Toll/IL-1 receptor (TIR) domain. Following ligand recognition and binding by the LRR domain, a signal is transduced across the membrane to the TIR domain, which activates associated adaptor proteins and subsequent signal transduction cascades. The detection of invading microorganisms by Toll-like receptors results in the induction of reactive oxygen and nitrogen intermediates as well as acquired immune responses [97]. Currently, there are 10 human and 9 murine TLR's known to exist within the mammalian TLR family which recognize different components of microbes [98]. The LRR domains of TLR2 and TLR5 have been shown to bind peptidoglycan (PG) and flagellin [99]. A single bacterium is able to activate multiple TLRs such as TLR4 by LPS, TLR5 by flagellin, or TLR2 which recognizes PG [100].

1.4.1.2. Complement

The complement system is a defence mechanism that generates effector molecules involved in both innate and acquired immunity. Complement activation is mediated through three different pathways including the classical, the alternative and the lectin pathway. Their effector functions include lysis of bacteria, opsonization and the attraction of phagocytes to the site of complement activation [101]. Complement is activated through three different pathways and all merge together in the activation of C3 convertase which is a surface associated enzyme complex that cleaves C3. Cleavage of C3 activates deposition of C3b on the surface of

microorganisms and release of C3a which is a potent chemoattractant. C3b and its by product C3bi, are deposited on the surface of microorganisms which results in their engulfment by phagocytic cells.

Evidence has been presented that suggests a significant proportion of HUS patients have mutations in genes encoding proteins belonging to the alternative complement pathway [102]. The alternative pathway provides an antibody-independent means of clearing bacterial infections by coating them with C3b thereby activating the terminal complement pathway. Many different proteins including Factor H, Membrane cofactor protein and Factor I are involved in this process and mutations in these proteins often impair the alternative pathway suggesting a possible role contributing to the pathogenesis of HUS.

1.4.1.3. Antimicrobial Peptides

Antimicrobial peptides are small polypeptides that have an antimicrobial activity and are used by all eukaryotic organisms including plants, insects and animals [103]. They are synthesized and secreted by polymorphonuclear leukocytes, macrophages and mucosal epithelial cells. Major antimicrobial peptides known to exist in cattle include: defensins, cathelicidins and anionic peptides [104]. There are several cationic antimicrobial peptides and a few groups of anionic antimicrobial peptides in domestic animals [301]. The defensins (α , β and θ) are approximately 2-6 kDa in size and are cysteine containing cationic peptides possessing three disulfide bonds [105]. The cathelicidins all possess an N-terminal signal peptide called the cathelin prosequence [103]. Cathelicidins are α -helical peptides and have microbicidal activity against Gram-negative and Gram-positive bacteria [103]. The peptides have been demonstrated to bind to LPS and provide protection in animal models against bacterial infection [106]. Other mammalian antimicrobial peptides include histatins and dermicidin [107]. Antimicrobial peptides have different mechanisms of killing pathogens as well as different ways to stimulate the immune system. Immunostimulatory functions include induction of chemotaxis of immature dendritic cells and T cells, activation of antigen-presenting cells (APC), increased glucocorticoid production, macrophage phagocytosis, mast cell degranulation, complement activation, and IL-8 production [108, 109].

1.4.2. Adaptive Immunity

In 1938, Tiselius and Kabat [107,[110] used electrophoresis to show that cell-free serum from an animal immune to a certain toxin contained more material in the γ -globulin peak than the serum from a non-immune animal [110, 111]. This was the first identification of antibodies and the cell-free specific resistance referred to as humoral immunity.

It was shown in 1960 that animals depleted of their lymphocytes became immunodeficient. Immunocompetence was re-established upon reconstituting the animals with lymphocytes from a syngeneic donor (genetically identical member of the same species). This established the concept of cell mediated immunity (CMI) and confirmed that lymphocytes are indeed the cells responsible for mounting an immune response [58]. Another key revelation around this time was that lymphocytes express positive memory. The same animal mentioned above was also reconstituted with cells from a syngeneic donor already exposed to a particular antigen. The animal mounted an immune response that was characteristic of a secondary immune response [58]. Secondary immune responses are generated faster than a primary immune response and have increased magnitude [91].

1.4.2.1. Antigen Presentation

There are two major classes of lymphocytes based on their origin and surface molecules. When an immature stem cell migrates from the bone marrow to the thymus, a primary lymphoid organ, a thymus-dependent lymphocyte (T cell) is generated. T cells in mice, bear the Thy1 antigen [112]. On the other hand, if an unspecialized stem cell differentiates in the bone marrow (another primary lymphoid organ), a B cell arises. B cells bear surface immunoglobulins (sIg). B and T lymphocytes bear receptors on their cell surface which are antigen specific [58]. T cells possess a T cell receptor (TcR) which is MHC restricted. Briefly, different MHC molecules are found on different cell types. Overall, MHC class I molecules are found on all nucleated cells and MHC class II molecules are found on B cells, macrophages and dendritic cells [113]. Therefore, the TcR is specific to the antigen in the context of an MHC molecule [112]. Surface immunoglobulins on B cells serve as the receptor and are able to bind antigen directly (not MHC restricted). When a B cell binds an antigen, this triggers the B cell to develop into antibody-forming plasma cells. Since B cells produce antigen-specific receptors with a single specificity,

the resulting antibodies secreted by the plasma cell have identical binding specificity to the original receptor. A large pool of plasma cells is generated by the B cell in order to increase the amount of antibody that is necessary to combat infection successfully [58]. This process takes time and therefore it is usually quite a few days before antibodies are detected following primary contact with an antigen.

B and T lymphocytes leave primary lymphoid organs and enter the blood, circulating throughout the body searching for foreign antigens. Some leave the blood and enter the secondary lymphoid organs such as the spleen, lymph nodes and Peyer's patches. Lymphocytes possess "homing receptors" that determine which secondary lymphoid organ the lymphocyte will emigrate to. Secondary lymphoid organs also bear ligands called addressins that recognize the specific homing receptors [58]. Histological staining of lymph nodes showed that B cells primarily inhabit the cortex whereas T cells are located mostly in the paracortex [58]. Peyer's patches are specialized lymph nodes located in the mucosa of the gastrointestinal tract containing both B and T cells [114]. Some researchers have speculated that the role of the Peyer's patches in young calves differs along the length of the gastrointestinal tract. For example, the Peyer's patches in the ileum serve as a source of naïve B cells and the role of the Peyer's patches in the jejunum act as a site of immune induction [115]. In the spleen, the white pulp region is the site of immune responses where both B and T cells reside [92].

1.4.2.2. Activation of T cells

T lymphocytes can be further divided based on their surface antigens and restriction specificity. Many surface markers have been identified using monoclonal antibodies hence the T lymphocytes can be divided into the $\alpha\beta$ CD4⁺ helper T lymphocytes, $\alpha\beta$ CD8⁺ cytotoxic T lymphocytes, $\gamma\delta$ T lymphocytes and CD4⁺, FOXP3⁺, CD25⁺ regulatory T lymphocytes [116]. CD4⁺ T cells respond to antigen by proliferating and are MHC class II restricted. This means that MHC class II bearing cells (commonly referred to as accessory cells) are required for CD4⁺ T cell activation and proliferation [58]. Studies have shown that accessory cells "process" antigens before presenting it on MHC class II molecules. Later this "processing" was shown to be simply the breaking down of antigen into fragments [58]. Experiments using small synthetic peptides revealed that fragments derived from processing have two distinct sites. The epitope

site is in contact with the TcR and the agretope site binds to the MHC class II molecule [16]. Therefore, foreign substances must first be engulfed by the accessory cell, degraded inside the cell and their peptides displayed on the surface MHC class II molecules. CD8⁺ T lymphocytes are cytotoxic (CTL), lysing infected cells and are MHC class I restricted. The same process is required for the activation of CD8⁺ T cells except in MHC class I bearing cells. Therefore the primary function of MHC class I and II bearing cells is to present peptides to CD4⁺ T cells and CD8⁺ T cells respectively [58].

Peptides bound to MHC class II molecules are insufficient to cause activation of a peptide-specific resting CD4⁺ T cell [8]. Binding of the antigen-specific TcR to the peptide on an MHC-bearing accessory cell induces chemical changes within the T cell and this represents what is referred to as signal 1. In order for CD4⁺ T cell activation to be completed, the CD4⁺ T cell must receive signal 2. Signal 2 is generated from interactions between costimulatory molecules on the accessory cell and the CD4⁺ T cell [16]. The most well known costimulatory molecules are B7.1 and B7.2 which interact with CD28 on the CD4⁺ T cell [117]. Upon receiving signal 2, CD4⁺ T cells are fully activated. After receiving signal 2 CD4⁺ T cells increase expression of IL2 receptors on their surface. Interleukin 2 was originally called the T cell growth factor because it induces T cell proliferation [117].

This model of CD4⁺ T cell activation is somewhat incomplete. Many researchers believe that the activation of every lymphocyte requires the antigen-mediated interaction of the lymphocyte to be activated with a different antigen-specific “collaborator lymphocyte” [7]. This is supported by the fact that CD4⁺ T cell activation seems to require the presence of CD4⁺ T cells. This requirement also takes into consideration the need for self tolerance [118]. Accessory cells such as macrophages and dendritic cells take up antigen indiscriminately including self antigens [118]. Therefore a modified two signal model has been proposed that incorporates this theme. The current model is complicated as the interaction between the CD4⁺ T cell and the “collaborator lymphocyte” is indirect and involves a few steps [16]. For example, an immature dendritic cell located just below the gut epithelium encounters a foreign pathogen. The dendritic cell phagocytoses the pathogen and processes it for antigen presentation via MHC class II molecules. This induces chemical changes within the dendritic cell resulting in expression of a greater number of costimulatory molecules (B7.1 and B7.2) on its surface [7]. The dendritic cell

then migrates to a Peyer's patch (a secondary lymphoid organ) to present the pathogen's peptides to the "resting" cells of the adaptive immune response. First, a resting CD4⁺ T cell receives signal 1 and signal 2 from the mature dendritic cell. Signal 1 is received upon the TcR recognizing and binding to antigen in an MHC class II-restricted manner and signal 2 is received through the binding of costimulatory molecules [117]. The CD4⁺ T cell is still not fully activated and still requires the participation of an antigen specific "collaborator lymphocyte"[16]. Therefore, presentation of the antigen by a B cell also through MHC class II molecules to the CD4⁺ T cell has been proposed. This requires that the B cell already expresses a receptor specific for this pathogen and that the B cell have already been activated by effector CD4⁺ T cells [16]. A final stage in CD4⁺ T cell activation could be the proliferation in response to IL2 [7].

The current model for CD8⁺ T cell activation is quite similar but slightly less complicated as the antigen-mediated interaction of the CD8⁺ T cell with the "collaborator lymphocyte" is more direct. An accessory cell phagocytoses and displays antigen specific peptides in an MHC class II restricted manner to a CD4⁺ T helper cell. The accessory cell is activated to increase expression of costimulatory molecules on its surface that are required for the activation of resting CD8⁺ T cells [16]. Resting CD8⁺ T cells bind to the peptide displayed in an MHC class I restricted manner and are fully activated through the binding of the recently induced costimulatory molecules [118]. If the accessory cell is not activated to express costimulatory molecules, it will present antigens to resting CD8⁺ T cells but will not activate them [8].

Accessory cells such as dendritic cells need to become activated themselves in order to thoroughly activate lymphocytes. This is achieved through two main pathways. The first pathway involves the binding of costimulatory molecules on CD4⁺ T cells such as CD40 with dendritic cells (via CD40 ligand or CD40L), CD80 and CD86 [113]. The second pathway involves activation through PRRs such as the TLRs. Binding of PAMPs to PRRs activates dendritic cells to a more mature state which includes the expression of costimulatory molecules [113], MHC class II molecule presentation on the surface of the dendritic cells increases and dendritic cells cease to take up any other materials [113].

Therefore, it is hypothesized that accessory cells decide the type of cytokine profile to secrete based on the pathogen it encounters [113]. Based on the type of cytokines produced, the CD4⁺ T helper cell can stimulate either a cell-mediated (Th1 type) or humoral (Th2 type) immune response [119]. Interleukin 2 and IFN γ are considered as the dominant cytokines during the Th-1 response whereas IL4, IL5 and IL10 are characteristic of a Th2 response. However, it is known that in cattle and humans, IL-10 can be produced by Th1 and Th2 cells and regulate all their activities [120]. In an attempt to characterize bovine Th cell subsets, Brown et al [121] found parasite antigen-specific Th cell clones that co-expressed IFN γ and IL4.

1.4.2.3. B cell Activation

The activation of B cells also requires two signals. Signal 1 is produced upon antigen binding to the B cell receptor or surface immunoglobulin (sIg). The second signal is produced upon the binding of T helper cells (via the TcR) to antigenic peptides displayed by the B cell in an MHC class II restricted manner. Further studies revealed that resting B cells also have a ligand on their surface for CD40 called CD40L. The interaction of CD40L with CD40 from T helper cells is central to B cell activation. Signal 1 alone in the absence of the second signal has been shown to be paralytic.

1.4.2.4. T Regulatory Cells

CD4⁺, FOXP3⁺, CD25⁺ regulatory T cells are either thymus derived lymphocytes or peripherally activated lymphocytes that control peripheral immune responses to self and non-self antigens. The ability of the immune system to differentiate between self-antigens and non-self antigens is based on multiple events in the thymus and periphery and is important for the preservation of immune homeostasis. The immune system has evolved a variety of multistep mechanisms to attain self tolerance. These include two mechanisms of tolerance resulting in either deletion of self-reactive T cells or the induction of non-responsiveness. First is central tolerance which involves the clonal deletion of self-reactive T and B cells exposed to self antigens at the early stages of development [122]. Second is peripheral tolerance which is a mechanism by which self reactive T lymphocytes are prevented from causing autoimmune diseases [123].

1.4.2.5 Adaptive Immune Responses to STEC

To date, the majority of studies done on adaptive immune responses to STEC have been limited to studies done with A/E pathogens using the *Citrobacter rodentium* model. Studies have shown that mice lacking acquired immunity, such as RAG-1- knockout mice deficient in mature T and B lymphocytes, were unable to clear infection with *C. rodentium*. In contrast, infection of wildtype mice leads to an inflammatory response with full recovery within 3 weeks [124]. Initially the RAG1 knockout mice showed a similar response to the wildtype, but within two weeks, the outcomes were dramatically different. This suggests that the innate system is initially important in controlling the infection and the adaptive response may be necessary to resolve the infection. Interestingly, IFN γ production was raised substantially in the wildtype mice which was in contrast to the IFN γ production in the RAG-1- knockout mice which dropped below uninfected level [125].

Other studies have shown that mice depleted of CD4+ T cells do not clear infection with *C. rodentium*. It was suggested that the reduced number of CD4+ T cells were unable to assist B cells causing a reduced antibody response. The use of μ MT mice, which have no IgG+ or IgM+ B cells were shown to be extremely vulnerable to *C. rodentium* infection [126]. This susceptibility to systemic immunity was reversed by adoptive transfer of immune sera, suggesting that antibodies play a crucial role during infection [127].

1.4.3. Bovine Mucosal Immunity

The intestinal immune system plays an important role in preventing disease as it represents a massive interface between the body and the external environment. A variety of cell types belonging to the innate and adaptive immune systems are devoted to recognizing, presenting and/or removing foreign microorganisms within the GI tract. The mucosa-associated lymphoid tissue (MALT) is distributed around the body for effective antigen sampling as it is the initial inductive site for mucosal immunity (Table 1) [128]. Structural features of the MALT include lymphoid tissue that is physically adjacent to the mucosal surface [128]. MALT also possesses organized structured lymphoid tissue. This includes lymphoid follicles and interfollicular areas [128]. The epithelium overlying the MALT contains lymphocytes and cells specialized in the uptake of antigen (called M cells) [128]. Antigen sampling in the MALT

occurs only through M cells [128]. And finally, the high endothelial venules (HEV) are used by recirculating lymphocytes to enter the MALT [128]. The MALT is subdivided based on location within the body to include:

CALT	Conjunctiva-associated lymphoid tissue
SALT	Salivary gland-associated tissue
LADLT	Lacrimal drainage-associated lymphoid tissue
NALT	Nose-associated lymphoid tissue
LTALT	Larynx-associated lymphoid tissue
BALT	Bronchus-associated lymphoid tissue
Gastric MALT	Gastric mucosa-associated lymphoid tissue
GALT	Gut-associated lymphoid tissue

Table 1. Mucosa-associated lymphoid tissue distribution within the body. Locations in the body where the MALT is situated in order to effectively sample antigens and thus induce appropriate immunological responses (both adaptive and innate) to foreign material and invading pathogens.

1.4.3.1. Innate Immunity

The innate immune system in the GI tract is particularly important because it is first to come into contact with invading microorganisms and hence is aimed primarily at preventing these microorganisms from getting access to the intestinal epithelium. The intestinal epithelium consists of a single cell layer joined firmly together by tight junctions, highly regulated macromolecular protein formations [129]. Tight junctions are composed of numerous proteins including occluding and claudins and form a physical barrier against the penetration of invading

microorganisms [129]. The intestinal epithelial cells also physically impede pathogen attachment through their brush-border microvilli. This physical hindrance is reinforced by mucus and glycocalyx that is continuously produced by goblet cells which are present in the crypt and villus epithelium of the GI tract. This polysaccharide network traps invading microorganisms which are subsequently eliminated through gut peristalsis and hydrous flow from the digestive tract [93]. Trefoil peptides (pS2, SP and ITF), also produced by goblet cells, are secreted in response to intestinal injury [129]. Trefoil peptides have been shown to protect the epithelium from bacterial products such as toxins. Antimicrobial peptides such as defensins and cathelicidins are also produced by the intestinal epithelium to combat invading microorganisms.

The innate immune system also directly senses invading microorganisms through PRRs. Stimulation of PRRs with bacterial products, such as LPS or pro-inflammatory cytokines, such as IL-1 β and TNF- α , activates signalling cascades that lead to inducible nitric oxide synthase (iNOS) transcription. This pathway results in the activation of p38 mitogen-activated protein kinase (MAPK), NF- κ B and Janus-activated kinase–signal transducer and activator of transcription–interferon regulatory factor 1 (JAK–STAT–IRF1) pathways [130, 131]. In the GI tract, two PRRs are particularly important including the TLRs (mentioned previously in section 1.4.1.1) and the Nod-like receptors (NLRs). The NLRs are divided into sub-families based on the composition of their N-terminal domain [93]. Nod1 and Nod2 recognize bacterial peptidoglycan and in turn stimulate NF- κ B resulting in the induction of pro-inflammatory cytokines [93]. There are at least 10 known mammalian TLRs each recognizing their own unique agonists for example, TLR4 recognizes bacterial LPS and TLR5 recognizes bacterial flagellin [93]. Toll-like receptor signalling results in the induction of anti-microbial peptides and pro-inflammatory cytokines as well as the activation of dendritic cells. Far less information is available for bovine TLRs than for rodent and human TLRs. To date, the bovine homologues for TLR2 and TLR4 have been cloned and partial sequences exist for TLR3 and TLR9 [98]. Toll-like receptor 2 is functionally associated with TLR1 and/or TLR6. The TLR2/TLR1 heterodimer recognizes triacylated lipopeptides, whereas the TLR2/TLR6 recognizes lipoproteins and lipoteichoic acid (LTA), found in the peptidoglycan layer of Gram-positive bacteria [132]. Binding of LPS to TLR4 results in the recruitment of MyD88 (myeloid differentiation primary

response gene 88) through the interaction of TIR-homology domains in TLR4 and MyD88 [133]. Flagellin, a bacterial virulence factor, is recognized by TLR5 present on the basolateral side of the epithelial cell [134] whereas TLR9 is present in the endosome and recognizes unmethylated bacterial cytosine–phosphate–guanosine (CpG) motifs [135].

1.4.2.2. Cellular Immunity

The gastrointestinal-associated lymphoid tissues (GALT) represent the largest immune compartment within the body. The GALT prevent pathogens from invading the mucosal tissues and recognizes commensal organisms that are required for effective food digestion and processing. The GALT has been extensively studied in sheep and cattle and is present as patches in the colon, jejunum, ileum, cecum and the rectum [128]. These patches were first identified in 1677 by Johann Conrad Peyer in sheep as “intestinal glands” and hence are referred to as Peyer’s patches [115]. The GALT is functionally divided into the inductive sites and the effector sites. The inductive sites include mesenteric lymph nodes, Peyer’s patches (in the small intestine) and isolated lymphoid follicles whereas the effector sites include the epithelium and the lamina propria. The lamina propria contains most aspects of the immune system including a large number of lymphocytes such as B cells, macrophages, dendritic cells and T cells [136]. The epithelium expresses TLRs such as TLR 2, 3, 4, 5, 7/8 and 9 [100] and also contains a large number of T cells of which the majority are CD8+ [136]. The bovine GALT develops at the fetal stage and jejunum Peyer’s patches (JPP) can be observed in five month old foetuses increasing in number during foetal life and remaining throughout the entire lifespan of the animal. Ileal Peyer’s patches (IPP) however develop later in seven month old foetuses growing remarkably large and accounting for 80-90% of the total mass of Peyer’s patch tissue [128, 137]. By the age of two years, both bovine and sheep IPP undergo an age-dependent involution and isolated lymphoid structures have taken their place [128].

Peyer’s patches are areas of organized lymphoid tissue that contain follicle centers and well-defined cellular zones covered by the follicle-associated epithelium (FAE) [138]. The FAE is distinct in that it does not contain any goblet cells or secretory component but does possess shorter villi and specialized membranous (M) cells [114]. M cells function as antigen-sampling cells primarily transporting antigens to the sub-epithelial dome of Peyer’s patches which

subsequently primes underlying lymphocytes for an immunological response. M cells are characterized by having irregular surface microvilli and a basolateral cytoplasmic invagination. This invagination creates a pocket permitting lymphocytes and macrophages from the sub-epithelial dome to enter assisting the initiation of an immune response [114]. It has been hypothesized that M cells develop from undifferentiated dome-associated crypt cells in the presence of lymphocytes [114]. The sub-epithelial dome area contains plasma cells, dendritic cells, macrophages and B cells.

Ruminant IPP and JPP have distinct differences in their architecture, lymphocyte organization and life span [139]. B cell development in cattle occurs predominately in IPPs and therefore it is considered to be a primary lymphoid organ [115]. Ruminant IPPs develop into a single continuous aggregation of lymphoid follicles often reaching two meters in length [137]. Approximately one month after birth, cattle IPPs have numerous long, densely packed oval lymphoid follicles that contain almost all B cells with very few CD4⁺ and CD8⁺ T lymphocytes as well as small sub-epithelial domes and interfollicular regions [137]. Rather, B cell lymphopoiesis occurs in the IPP at a very high rate generating approximately 3.6×10^9 cells/hour supplying B cells to the periphery most of which possess surface IgM [115, 137]. Studies have shown though that only 5% of newly produced B cells actually migrate to the periphery as many die *in situ* by apoptosis. This on-going selection within IPPs still produces 5×10^7 cells/hour which is 10 times greater than from JPPs [115]. Jejunum Peyer's patches, on the other hand, are thought to be secondary lymphoid organs possessing large interfollicular regions and small pear shaped lymphoid follicles [140]. Jejunum Peyer's patches occur as numerous distinct accumulations of lymphoid follicles often ranging in number depending on the species [137]. Jejunum Peyer's patches contain approximately 25% mature CD4⁺ T lymphocytes whereas IPPs contain only 1% CD4⁺ T cells [115]. One study showed that injection of antigen into intestinal loops containing JPPs resulted in the formation of specific antibody forming cells and antibody titres indicating the JPPs are an efficient site for the induction of a mucosal immune response [115]. Both IPPs and JPPs are encapsulated and surrounded by lymphatic sinuses that are in turn connected to lymph vessels. These lymph vessels drain the intestinal villi and surrounding sinuses thus continuously bathing PP follicles in lymph [115].

1.5. Treatment, Control and Prevention of STEC

Treatment options for STEC infection can be categorized into three main areas including i) infection prevention ii) therapies directly targeting Stxs and iii) therapies targeting downstream molecular events [141]. Supportive therapy as well is necessary but often does not prevent microvascular injury or CNS and other organ complications.

1.5.1. Treatment of STEC

More than 76 million people within the United States become ill from the consumption of contaminated meat products [142]. Therefore strategies aimed at reducing pathogenic microorganisms within meat products in order to reduce human exposure and thus decrease human foodborne illnesses have gained much attention.

1.5.2. Control and prevention in humans

Shiga toxin-producing *E. coli* is responsible for 70-90% of HUS within children worldwide [141]. Therefore supportive treatment focuses on abolishing complications related to four main systems within the body including gastrointestinal, haematological, vascular and renal. Antimotility agents (drugs used to reduce gastro-intestinal motility) are not recommended as they have been associated with an increase in HUS. As well, narcotics and nonsteroidal agents are also not recommended but morphine and acetaminophen are used to manage abdominal cramping and pain. Volume expansion by IV rehydration with isotonic solutions has been shown to provide renal protection and stop STEC-induced HUS if administered within the first four days of diarrhea [141]. Approximately 80% of HUS patients require packed red blood cells to combat symptomatic anemia. Also, dialysis has been found to improve morbidity by correcting severe electrolyte imbalances and fluid overload. Hemo- and peritoneal dialysis has been shown to be equally effective and needs to be continued until recovery.

The use of antibiotics to treat STEC infections is a very important clinical matter. It has been suggested that specific outcomes may be affected by the STEC strain, phage-encoded toxin genes, type of antibiotic administered as well as timing and concentration of antibiotic [141]. A Japanese study in 1999 revealed that administering fosfomycin during the first 2 days of illness reduced the incidence of HUS. The specific strain, termed the Sakai strain, was shown to

express both Stx1 and Stx2. Treatment during days 3-5 though showed no protective effect [143]. Two retrospective studies in Minnesota and Washington State showed no association between the use of antimicrobials and the development of HUS [144, 145]. In contrast, Wong et al [146] in 2000 showed an increase in the development of HUS with antibiotic treatment. The design of the experiment ensured that only individuals likely to develop HUS were administered antibiotics. Because of this study, many researchers recommend only supportive care during STEC infection. Many *in vitro* studies have also revealed an increase in Stx production in the presence of certain antibiotics. Thus, antibiotics interacting with bacterial DNA including fluoroquinolones and β -lactams which induce bacteriophage replication, lead to an increase in Stx production.

The oral administration of Stx-binding agents has been extensively studied as a treatment option for STEC infection. One study performed in mice showed that administration of a polymer of Gb₃ protected all the mice from a fatal dose of STEC as well as reduced serum levels of Stxs [147]. Another study done in 2004 using a synthetic toxin binder called Synsorb Pk showed no reduction in HUS or serious extra-renal complications in patients when administered immediately after the diagnosis of HUS compared to the placebo group. Various hypotheses exist to explain the failure of the multicenter clinical trial including the timing of the agent and the low affinity of the agent for the polyvalent toxin [148]. More recent multibranched Stx receptor analogues have been developed and their therapeutic potential is being considered. One example of this is an oligovalent, water-soluble carbohydrate ligand called STARFISH developed by a Canadian research team. Crystallography of STARFISH binding to Stx showed all five B subunits simultaneously bound [149]. The intravenous administration of monoclonal antibodies to Stxs has also been considered. Studies in mice and gnotobiotic piglets have shown that monoclonal antibodies to Stxs are protective against further oral STEC challenge [150, 151]. Akiyoshi et al [152] have produced a human monoclonal antibody to the A subunit of Stx2 which awaits further investigations.

The last treatment option that targets downstream molecular events has been the least developed. Fu et al [153] demonstrated that FR167653 (a p38 inhibitor) reduced plasma levels of proinflammatory cytokines such as TNF α and IL-1 which decreased renal failure in rats. TNF α and IL1 are most likely involved in the apoptosis of glomerular endothelial cells and

inhibition of these proinflammatory cytokines may prevent HUS [153]. Other studies aimed at reducing proapoptotic signals or blocking the thrombotic cascade are ongoing [141]. Raife et al [154] have shown using greyhounds that pretreatment with lepirudin prevented the development of kidney lesions in two thirds of the test animals suggesting that inhibiting thrombin formation will also prevent the development of HUS.

1.5.3. Control and prevention in animals

Cattle are the primary non-human reservoir for STEC and it had been shown that 19% of retail uncooked beef is contaminated with STEC in the United States [155]. A global assessment of the STEC problem in cattle was done by Hussein et al [156] in 2007. They showed that over 50% of ground beef and over 40% of whole beef carcasses were contaminated with *E. coli* O157:H7 in the United States [10]. Of the 162 STEC serotypes identified, 43 have been isolated from HUS patients. Therefore much effort has been put into reducing the survival and transmission of STEC by focusing on i) farm management and disinfectant practices and ii) antibiotics, probiotics and bacteriophage use within cattle to increase cattle resistance to STEC infection.

Preventing cattle from coming into contact with STEC is tremendously difficult [157]. Therefore on-farm interventions have been exploited including chlorination of water, general disinfection of living quarters, switching grain-fed cattle to an all hay diet prior to slaughter and quarantining animals until they test negative [157]. General disinfection and chlorination of water produced less than desirable results and quarantining animals proved impractical due to the transient nature of faecal shedding [155]. Russell et al [158] in 2000 found that switching cattle to a hay diet resulted in an increased number of less acid-resistant *E. coli*. These less acid-resistant *E. coli* are hypothesized to be less likely to survive the acid environment of the human digestive barrier.

The use of antibiotics has been considered but concerns over bacterial resistance have prevented the issuing of a license allowing their use in cattle to control STEC. A promising study using a strain of *Lactobacillus* in cattle as a probiotic showed significant reduction in gut colonization and faecal shedding leading to further studies using *Enterococcus* and non-pathogenic *E. coli* [155]. Several studies have been done using bacteriophages to reduce *E. coli*

in cattle. Initial experiments were done using EPEC and studies showed that bacteriophage treatment of cattle reduced diarrhea and splenic EPEC colonization in calves [159]. Then Kudva et al were able to isolate *E. coli* O157-specific bacteriophages and demonstrated complete lysis of *E. coli* O157:H7 cultures *in vitro* [160]. Also treatment with *E. coli* O157:H7-specific bacteriophage reduced numbers of *E. coli* O157:H7 within the gastrointestinal tract of sheep experimentally infected with *E. coli* O157:H7 [161].

1.5.4. Vaccines

Since cattle are linked with human STEC infections, they are a logical vaccine target as a means to reduce the risk of human infections. In 2004 Potter et al [162] successfully reduced colonization of cattle by STEC by vaccinating with several type III secreted proteins such as Tir and EspA. These proteins play a role in colonization of the intestine. Vaccination consisted of three 2ml (50ug/ml) doses of supernatant secreted proteins. Results showed significant reduction in the numbers of bacteria shed in feces, the numbers of animals that shed, and the duration of shedding in an experimental challenge model [162].

A second vaccine trial was conducted where secreted proteins prepared from the wild type strain was compared to secreted proteins from a Tir mutant. Three groups of yearling cattle were immunized on days 0, 21, and 35, followed by oral challenge with *E. coli* O157:H7 on day 49 [162]. Vaccination with proteins from the Tir mutant was not as effective as those from the wild type strain. However, vaccination with proteins from the mutant provided more protection than the placebo. From day 2 post-infection onwards, 78.3% of the placebo group shed *E. coli* O157:H7 for at least 1 day as compared to 30% for cattle vaccinated with the Tir mutant vaccine.

In 2005, Peterson et al [163] performed a single commercial feedlot trial also to evaluate the effects of vaccinating cattle with type III secreted proteins. Four vaccination treatments were done including no vaccination, vaccination on day 42, vaccination on days 0 and 42 and vaccination on days 0, 21 and 42. The vaccine was administered sub-cutaneously in the neck of 480 steers, with 120 animals per group. Results of the trial showed that vaccination reduced the proportion of cattle shedding *E. coli* O157:H7 in their feces.

Recently a study done by McNeilly et al [164] demonstrated how systemic vaccination of cattle with H7 flagellin reduced the colonization of STEC. This vaccination resulted in high

levels of serum IgG, high levels of IgA in nasal secretion and measurable levels of both IgA and IgG in rectal secretions. In another study, Dean-Nystrom et al showed that vaccination of dams using intimin was capable of protecting against challenge with STEC O157 [165]. Other potential vaccines have been tested using intimin as an antigen. Agin et al [166] vaccinated rabbits with a STEC strain containing a non-functional intimin protein that resulted in protection against further STEC challenge. A study done in mice using a plant-based vaccine which included the carboxy-terminal cell binding domain of intimin, was proficient in reducing fecal shedding upon further challenge with STEC O157 [167]. Although vaccine trials using intimin as an antigen has shown promising results, a cross-protective vaccine based on this protein would be challenging because many different variations have been identified within STEC serotypes [168].

One of the most recent vaccine trial published in 2009 by Smith et al [169] used 3683 cattle and type III secreted proteins in which cattle were vaccinated on days 0 and 42. To detect shedding *E. coli* O157:H7, the terminal rectal mucosa was swabbed 3-5cm proximal to the rectoanal juncture. Results showed only 11 of 382 vaccinated animals were positive for *E. coli* O157:H7 revealing that vaccinated cattle were colonized significantly less with *E. coli* O157:H7 than nonvaccinated cattle [169].

Another very recent vaccine trial published in 2009 by Thorton et al [170] used the outer membrane siderophore receptor of *E. coli* O157:H7 to reduce fecal shedding in cattle. Two groups of fifteen cattle were used in the trial and were vaccinated on days 0 and 21 with either a placebo or the siderophore vaccine. Antibody titres to the siderophore protein were analyzed weekly from both groups. Two weeks after the second vaccination, cattle were challenged with *E. coli* O157:H7 and fecal samples and rectoanal swabs were taken to monitor bacterial shedding. Results of the trial showed that the vaccinated group had a significantly higher antibody titre to the siderophore protein and decreased *E. coli* O157:H7 fecal shedding as well as reduced the number of cattle shedding [170].

Several vaccine trials have been done in mice including a trial done by Gu et al [171]. They constructed a trivalent recombinant protein consisting of EspA, the C-terminal end of intimin and the B subunit of Stx2. Six-week old BALB/c mice were divided into seven groups each vaccinated with a different combination of recombinant proteins on days 0, 7 and 14

and then challenged with *E. coli* O157:H7. Gu et al [171] concluded from the trial that the recombinant proteins induced strong humoral responses to the recombinant proteins and vaccinating with combinations of recombinant proteins was more effective than vaccinating with individual proteins alone. Another recent vaccine trial in mice used two nonfused polypeptides consisting of the B subunit of Stx2 and the last 32 amino acids of the A subunit of Stx2. Antibody responses to Stx2 were produced as well the anti-Stx2 antibodies neutralized toxin *in vitro* and subsequently conferred partial protection against Stx2 *in vivo* [172].

2.0 Hypothesis, Objectives and Specific Aims of this Study

2.1. Hypothesis

We hypothesized that Shiga toxin 2 acts as an immunomodulator in cattle by downregulating immune responses which contributes to persistent colonization.

2.2. Objective

To date, little is known about STEC pathogenesis in cattle, in particular the virulence factors associated with persistence. Bacterial pathogens use a variety of mechanisms including toxins, in order to avert immune responses and successfully colonize their hosts [46]. This has been reported for STEC but the mechanism has yet to be defined. Therefore the primary objective of this project is to determine the effect of Shiga toxin 2 on bovine immune responses during STEC infection.

2.3 Specific Aims

Aim 1: To analyze lymphocyte function in the presence of purified Stx2.

Aim 2: To compare cytokine gene expression profiles *in vitro* using a bovine intestinal epithelial cell line in the presence and absence of purified Stx2.

Aim 3: To analyze bovine lymphocyte populations and cytokine profiles in the presence (wild type Stx2-producing STEC strain) and absence (Δ stx STEC mutant strain) of Stx2 *in vivo*.

Aim 4: To measure bovine immune responses during active immunization in the presence (secreted protein preparation isolated from a wild type Stx2-producing STEC strain) and absence of Stx2 (secreted protein preparation isolated from a Δ stx STEC mutant strain).

3.0 General Materials and Methods

3.1 Bacterial strains and growth conditions

Bacterial strains used in this study include *E. coli* wild type Stx2⁺ strain 1961 and an isogenic mutant Stx2⁻ strain 92192 which were kindly provided by Dr. Carlton Gyles (University of Guelph, Guelph, Ontario, Canada). The strains were stored at -70°C in 30% glycerol and grown in Luria-Bertani (LB) agar and LB broth (DIFCO, Becton-dickinson, Sparks, MD, USA) at 37°C with shaking. Strain #92192 is gentamicin resistant and was therefore grown in LB agar and LB broth plus 15µg/ml gentamicin (Gibco).

3.2 Isolation of specific cells of the immune system

3.2.1 Isolation of bovine peripheral blood mononuclear cells (PBMC)

Blood was collected from the jugular vein of Holstein cows in 0.3% EDTA (Sigma #431788) and centrifuged at 1400x for 20 minutes at room temperature. The buffy coat cell layer was removed and transferred to a new tube. The volume was adjusted with PBS (0.1M phosphate-buffered saline) + 0.1% EDTA and layered onto Ficoll-Hypaque (GE Health Care Biosciences AB, Uppsala, Sweden). The samples were centrifuged for 20 minutes at 2000xg. Cells at the Percoll interface were collected and transferred to a new tube and the volume adjusted with ice cold PBSA (137mM NaCl, 2.7 mM KCl, 4.3mM Na₂HPO₄·7H₂O, 1.4mM KH₂PO₄) + 0.1% EDTA. The samples were centrifuged for 8 minutes at 325xg at 4°C. The supernatant was decanted and the pellet resuspended in PBSA. The samples were washed with PBSA two more times at 4°C. Cell number was determined with a haemocytometer microscopically and viability measured using Trypan Blue [173] (Gibco #15250-061).

3.2.2 Isolation of bovine intestinal lymphocytes (IL)

Gut specimens were obtained from healthy 6-week old Holstein calves and transported to the lab in ice cold CMF-HBSS (Calcium- and Magnesium- free Hank's balanced salt solution, 5.4mM KCl, 0.3mM NaHPO₄·7H₂O, 0.4mM KH₂PO₄, 137mM NaCl, 5.6mM glucose, pH 7.4) /5% FBS (Seracare)/antibiotic/antimycotic (Gibco) solution. The gut tissue was cut into 10cm lengths and opened lengthwise. The tissue was then cut into 5cm² pieces and washed in an

environmental shaker for 15 minutes in CMF-HBSS/5% FBS/antibiotic/antimycotic/10mM DTT (Dithiothreitol) (Sigma #9760) at 37°C. The tissue pieces were then removed and placed into a new flask containing CMF-HBSS /5% FBS (Seracare) /antibiotic/antimycotic (Gibco)/ 10mM EDTA for 60 minutes at 37°C. After 60 minutes, the tissues were washed for 3 hours in CMF-HBSS /5% FBS/ 200U/ml collagenase 4 (Cedar Lane #LS004189, 200U/ml)/ 30ug/ml DNase I (Sigma #4965, 185000KU/ml) changing the solution after 60 and 120 minutes and reserving all supernatants. Each 50mls of supernatant was centrifuged at 300xg for 8minutes and the cell pellet resuspended in 10ml PBSA. This was layered onto Ficoll-Hypaque and centrifuged for 20 minutes at 2000xg. The buffy coat was collected and washed three times in PBSA + 5% FBS. Cell numbers and viability was confirmed using Trypan Blue (Gibco #15250-61).

3.3 Isolation of secreted proteins

Type III secreted proteins from both STEC strains were prepared as described [174]. A single colony of wild type Stx2⁺ *E. coli* O157:H7 and mutant Stx2⁻ *E. coli* O157:H7 strains (described in Section 3.1) were picked from LB plates, inoculated into 20mls of LB broth (or LB broth + 15µg/ml gentamycin) and allowed to grow at 37°C to stationary phase overnight. Both the Stx2⁺ wild type *E. coli* O157:H7 and the Stx2⁻ mutant *E. coli* O157:H7 overnight cultures were subcultured the next morning by diluting 100 fold in M9 minimal medium (Sigma-Aldrich, St. Louis, MO, USA) [175] supplemented with 0.5M MgSO₄ + 5% casamino acids + 0.5% glucose + 1% NaHCO₂. The cultures were then incubated using constant aeration at 37°C in a 5% CO₂ environment to an absorbance at OD₆₀₀ of approximately 0.7 [174]. The cultures were centrifuged at 5000 rpm for 20 minutes at 4°C and the pellets discarded, this was repeated two times [174]. The supernatants (containing the secreted proteins) were concentrated using a 10K Amicon filter (Fischer #13721) at 4°C with nitrogen gas. The filter was pre-soaked in sterile water overnight in a glass beaker. The preparations made using the 10K Amicon filter were used to confirm the wild type Stx2⁺ strain and the mutant Stx2⁻ *E. coli* O157:H7 strains (Chapter 6.0) and for the secreted protein trial (Chapter 7) [174]. This procedure was repeated three times so a total of 1.5 litres of culture supernatant was concentrated. The concentrations of the resulting secreted protein preparations were determined using the Bio-Rad DC Protein Assay.

3.4 Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of secreted protein extracts was carried out as follows. First, total protein was mixed with the same volume of 2X protein loading dye (100mM Tris HCl, 200mM dithiothreitol (DTT), 4% SDS, 0.2% bromophenol blue, 20% glycerol) and the mixture was loaded into individual lanes of a 12% SDS-PAGE. The proteins were separated at 120 volts in a gel apparatus (Model 250/2.5 Bio-Rad) for an hour and visualized following staining with Coomassie Brilliant Blue (20% MeOH and 10% Glacial Acetic acid and 0.5 g/L of Coomassie Brilliant Blue) for a period of 30 minutes and destained (20% MeOH and 10% Glacial Acetic acid) for 2 hours with regular buffer exchange [176]. For immunoblotting, the proteins were transferred from the gel to a nitrocellulose membrane (Bio-Rad) using a power supply (PowerPac200, Bio-Rad). Transferring the gel to a nitrocellulose membrane by electroblotting as recommended by the manufacturer (BioRad Laboratories) and included soaking the gel and nitrocellulose membrane in transfer buffer (3g/L Tris, 1.41g/L glycine, 200ml methanol, distilled water to a final volume of 1000ml, pH 9) for 5 minutes. The nitrocellulose filter was placed on top of the gel and two pieces of Whatman filter papers were placed on both the gel and the nitrocellulose sides. This was placed between two thick membrane supports and the whole assembly placed in a power supply chamber (PowerPac200, BioRad) and into a tank containing pre-chilled transfer buffer. The proteins were transferred at a current of 0.3mA for one hour. After the proteins were transferred to the nitrocellulose, the membrane was removed from the apparatus and the nitrocellulose membrane was placed in blocking solution (8.8g/L of NaCl, 0.2g/L of KCl, 3g/L of Tris base and 500 μ L of Tween-20, pH adjusted to 7.4) (TBS-T) with 3 % skim milk for 1 hour at room temperature. After incubation, the membrane was washed three times with TBS-T and incubated with a primary antibody for 1 hour at room temperature. After the incubation, the membrane was again washed three times with TBS-T and then incubated with an alkaline phosphatase-conjugated secondary antibody (1/2000) for one hour at room temperature. After the final antibody incubation, the membrane was washed once with TBS-T and once with alkaline (AP) buffer (12.11g/L of Tris base, 5.84g/L of NaCl, 1.04g/L of MgCl₂ 6H₂O, pH adjusted to 9.5). The membranes were then incubated for 15 minutes with AP buffer and

developed using nitroblue tetrazolium (NBT) salt (Sigma) and 5-bromo-4-chro-3-indolyl phosphate (BCIP) (Sigma) [177].

3.5 Cattle immunization protocols

Vaccines containing type III secreted proteins were prepared by formulating 500 µg of total protein with the adjuvant VSA3 as described [162]. Adult Holstein cows were immunized with 500µg hen egg lysozyme with or without Shiga toxin 2. Hen egg lysozyme was formulated with 30% VSA3 as the adjuvant and a total of 2mls administered subcutaneously in the neck of each animal. In each trial, vaccinations occurred on days 0 and 21. The serum samples were collected on days 0, 6, 10, 14, 21, 28 (35 if relevant) and serological responses were measured using an enzyme-linked immunosorbent assay (ELISA) as described [162]. Differences were considered significant at a P-value < 0.05 (Dunn's Multiple Comparison Test).

3.6 Cytokine Capture ELISA

The appropriate anti-cytokine specific monoclonal antibody was diluted (1/8000 for IFN γ , 8µg/ml for IL12, 1/2000 for IFN α , 1/1000 for TNF α , and 0.5µg/ml for IL10) and added to 96 round bottom plates (Thermo Labsystems #3655) and incubated at 4°C overnight. The plates were washed four times with TBST (TBS, 0.85% NaCl (w/v), pH7) containing 0.5% (v/v) of Tween 20). Two-fold dilutions of the samples were applied to the plate at 100µl per well in TBST-g (TBST + 0.1% gelatin). Each standard (see Table 2) was diluted in Fetal Bovine Serum (FBS) and serial two-fold dilutions were done for ten wells. The plates were incubated overnight at 4°C then washed four times with TBST. Each detection antibody was diluted in TBST-g (1/5000 for IFN γ , 1/1000 IFN α , 1/1500 for TNF α , and to 0.125µg/ml for IL10) and 100µl applied to each well for a minimum of one hour at room temperature. The plates were washed four times with TBST. The appropriate biotinylated antibody (Table 2) was diluted 1/10,000, added to each well and allowed to incubate for one hour at room temperature. The plates were washed four times with TBST. Streptavidin alkaline phosphatase (Jackson # 016-050-084; 50% glycerol) was diluted 1/5000 and 100µl was added to each well for a minimum of one hour at room temperature. The plates were washed four times with TBST. PNPP substrate (Sigma #N3254) was diluted in fresh PNPP buffer (1% diethanolamine, 0.5mM MgCl₂) to 1mg/ml and

Cytokine ELISA	Coating Antibody	Detection Antibody	Conjugate Antibody	Standard
IFNγ	Mouse anti rBoIFN γ monoclonal antibody 2-2-1A	Rabbit anti BoIFN γ 92-131 (1ng/ml)	Goat anti Rabbit IFN (H+L) biotin (Zymed #62-6140)	rBoIFN γ (2ng/ml)
IFNα	Mouse anti rBoIFN α Monoclonal antibody A2 (E2-1C6)	Rabbit anti BoIFN α 92-133	Goat anti Rabbit IgG (H+L) bitin (Zymed #62-6140)	rBoIFN α (1ng/ml)
TNFα	Mouse anti rBoTNF α monoclonal antibody 1D11-13	Rabbit anti BoTNF α Pool 88	Goat anti Rabbit IgG (H+L) biotin (Zymed #62-6140)	rBoTNF α (20ng/ml)
IL12	Mouse anti rBoIL12 CC301 (Serotec MCA 1782EL)	None	Mouse anti bovine IL12 CC326 biotin (Serotec MCA2173B) (5 μ g/ml)	rHuIL12 (Serotec PHP100) (200ng/ml)
IL10	Mouse anti rBoIL10 CC318 (Serotec MCA2110)	None	Mouse anti BoIL10 CC320 biotin (Serotec MCA2111B) (0.125 μ g/ml)	rBoIL10 (75U/ml)
IL4	Mouse anti bovine IL4 (Serotec MCA2371)	None	Mouse anti BoIL4 (MCA2372B) biotin (0.25mg/ml)	rBoIL4 (Serotec PBP006 20 μ g/ml)

Table 2. Cytokine-specific ELISA details. A description of the different antibodies used in each cytokine-specific ELISA. The last column is a description of the standard used (including starting concentration) during the ELISA procedure to generate a standard graph (Figure 11). The standard graph was used to extrapolate cytokine concentrations from supernatant isolated from each ligated loop. Note: the IL12, IL10 and IL4 specific ELISAs do not use a detection antibody.

r: recombinant

100µl added to each well. The reaction was stopped when the Optical Density (OD₄₀₅) of the first dilution of the standard curve reached an OD₄₀₅ of approximately 2.0.

3.7 Flow cytometry Analysis

After cell isolation, appropriate cell types were thoroughly resuspended in FACoLa (0.03% sodium azide and 0.02% gelatin in PBSA) and 100µl transferred to round bottom microtiter plates (1x10⁶ cells/well) (Nunc #449824). Fifty microliters of the appropriate monoclonal antibody (see Table 3) was added to each well and incubated on ice for 15 minutes. One hundred microliters of FACoLa was added to each well and the plates were centrifuged for 2 minutes at 350xg at a temperature of 4°C. The media/FACoLa was flicked off between washes and this was repeated two more times for a total of three washes (second and third washes used 200µl FACoLa). One hundred microliters of the appropriate fluorochrome conjugated secondary antibody (see Table 3) was diluted in FACoLa and added to each well and incubated on ice for 15 minutes. A total of three washes were repeated (second and third washes used 200µl FACoLa) and the plates were centrifuged for 2 minutes at 350xg. At the end, 200µl of 2% formaldehyde (in PBS) was added to each well to fix the samples. The plates were wrapped in tinfoil and stored in the refrigerator at 4°C until ready to be analyzed (no longer than 2 weeks). Ten thousand events were acquired for each sample using a FAC Scan (Becton-Dickinson, Mountain View, CA) flow cytometry and Cell Quest program. Negative controls included cells with media only. The amount of specific antibody binding was calculated by subtracting the population of cells that bound the isotype-matched irrelevant monoclonal antibody.

3.8 Statistics

Statistical analysis of the humoral and cell-mediated immune responses (non-parametric Mann Whitney U test) were performed using GraphPad Prism 5 (Graphpad Software, San Diego, CA). Differences were considered significant when P<0.05.

Cell Type	Primary Antibody	Secondary Antibody	Isotype Control Antibody
CD4+ T cell	CACT183A	FITC anti-mouse IgG1	IgG1
CD8+ T cell	CACT80C	FITC anti-mouse IgG1	IgG1
B cell	AbD Serotec CC21/MCA1424G	FITC anti-mouse IgG1	IgG1
Dendritic cell	CD11c: VMRD BAQ153A	FITC anti-mouse IgM	IgM
	MHC class II: VMRD H34A	PI anti-mouse IgG2	IgG2b
Gb ₃ + /CD77+ cell	CD77: Immunotech	FITC anti-rat IgM	IgM
Leukocyte positive populations	CD45: VMRD CACTB51A	FITC anti-mouse IgG2a	IgG2a

Table 3. Primary and secondary antibodies used during flow cytometry.

4.0 Functional Analysis of Shiga toxin 2 with Bovine Peripheral Blood Mononuclear Cells and Intestinal Peyer's Patches Lymphocytes *In Vitro*

4.1. Introduction

The shiga toxins represent a group of A-B cytotoxins in which there is one A subunit to every five B subunits. The B subunits are responsible for recognizing and binding to the receptor. In this case the receptor is a glycosphingolipid called Gb₃ [76]. After receptor binding, the A subunit is internalized into the cell and is responsible for completely truncating protein synthesis which induces cell death [58, 68]. Shiga toxins only cause disease in a limited number of hosts, including humans. In humans, symptoms can range from a mild diarrhea which is usually self limiting to the life threatening HUS. Kidney failure results from the cytotoxic effects of shiga toxins with a mortality rate of 3-5% [49].

The Stx2 family consists of Stx2, Stx2c, Stx2c2, Stx2d, Stx2e, and Stx2f [50]. Stx2 has been associated with a more severe outcome of disease than Stx1, and is about 1,000 times more toxic for human renal microvascular endothelial cells than Stx1 [49]. In a study of 255 strains, all 20 cases of HUS included either subtype Stx2, subtype Stx2c or the two combined [49]. As well experimental evidence using a primate animal model showed that administration of Stx1 failed to induce symptoms of HUS whereas the opposite was found with Stx2 [49].

The primary non-human reservoir for STEC is cattle. Contrary to what is observed in humans, cattle over the age of 3 weeks do not exhibit any apparent clinical symptoms of disease or histological lesions upon infection with STEC strains [70]. The reason(s) for the different outcomes upon infection with STEC in humans and cattle are unknown although there is an increasing amount of data suggesting that it may be in part due to the Shiga toxins [46]. In 2002, Stamm et al showed that bovine lymphocytes express a functional Gb₃ receptor [178]. A few years later, Menge et al showed that Stx1 binds to Gb₃⁺ lymphocytes and reduces their numbers *in vitro* after 4 days of incubation [68]. Lowered bovine lymphocyte proliferative responses are also seen *in vitro* in the presence of Stx1 [68, 179]. Therefore, aside from its known potent cytotoxic effects within humans, it has been hypothesized that the shiga toxins may possess additional immune modulating activities within the bovine host thus aiding in STEC

pathogenesis. In the present study, we determined *in vitro* the effects of Stx2 on bovine PBMCs and Intestinal Peyer's patches lymphocytes (IPPL) populations.

4.2. Materials and Methods

4.2.1 Cytotoxicity Assay

The purpose of the VERO cell cytotoxicity assay was to determine the concentration of purified Stx2 that kills half of the cell population in order to use an appropriate dose of Stx2 for future experiments. It is accepted that VERO cells are highly sensitive to Stxs and for this reason they were chosen for the assay [68]. The cytotoxic activity of the Stx2 preparation (Sigma Aldrich, V2513-10UG, 0.1mg protein/ml) [180, 181] was determined as described by Gentry et al [182]. Briefly, 50µl of a 10-fold dilution series of the Stx2 preparation was added to a 96 well plate already containing 50µl of cell culture media (RPMI 1640 (Invitrogen #11875101) containing 10% FBS (Seracare), 2mM glutamine (Sigma #25030081), 100U penicillin (Invitrogen), and 100mg/ml streptomycin (Sigma)). This was allowed to incubate for 30 minutes at room temperature. Then, 50µl of VERO cells at a concentration of 8×10^5 cells/ml were added to each well and incubated at 37°C under 5% CO₂ for 96 hours. Cellular metabolic activity was measured using the MTT Reduction Assay as described below. The percentage of cellular metabolic activity was calculated using the following formula:

$$[\text{OD (sample)} - \text{OD (positive control)}] / [\text{OD (negative control)} - \text{OD (positive control)}] \times 100.$$

The 50% cytotoxic dose (CD₅₀) value was determined using dose-response curves as the toxin concentration causing a 50% reduction in cellular metabolic activity [69] (Figure 2).

4.2.2 Flow Cytometry of bovine PBMC and IL populations

Flow cytometry was done on freshly isolated bovine PBMC and IL (from the ileal Peyer's patches and the jejuna lamina propria) to confirm the presence of the Stx receptor on the cell surface. Since the IL population is heterogeneous, cells were first incubated with a monoclonal antibody to CD45 (a leukocyte antigen) to distinguish the leukocytes from other cell types. This step was not done with the PBMC population. Twenty microliters of rat anti-CD77

monoclonal antibody (2mg/ml, Clone 3813, Immunotech) was incubated with 1×10^6 cells which were incubated on ice for 15 minutes and washed three times in FACoLa. Then, 100µl of FITC anti-rat IgM (100µg, eBioscience #11-0990-82) was added to each well. The preparation was allowed to sit on ice for 15 minutes and subsequently washed in FACoLa three times. The amount of specific antibody binding was calculated by subtracting the population of cells that bound the isotype-matched irrelevant monoclonal antibody.

4.2.3 Bovine PBMC and IPPL Viability in the presence of purified Shiga toxin 2

4.2.3.1 MTT Viability

The cellular metabolic activity was assayed by measuring the reduction of tetrazolium salts (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide). After cell isolation (protocols described in sections 3.2.1 and 3.2.2) bovine PBMCs and IPPL were resuspended in DMEM (Sigma #D6429) supplemented with 10% FBS (Seracare), 1% non-essential amino acids (Gibco #11140), 1% sodium pyruvate, 1% HEPES (Sigma #IMH0887), 0.1% gentamycin (Gibco #15750-060) and 0.1% mercaptoethanol. In stimulation assays, the media was additionally supplemented with concavalin A (ConA) (Sigma #C5275) at 5µg/ml or lipopolysaccharide from *E. coli* O111:B4 (LPS) (Sigma #L2630) at 25µg/ml.

Briefly, 10µl of MTT stock solution (5mg/ml in PBS) was added to each well of a 96-well plate. The reaction was incubated at 37°C for four hours and then stopped by the addition of 100µl of detergent reagent. The dye crystals were dissolved by the addition of 100µl of 10% SDS in distilled water. The plates were left at room temperature overnight and absorbance read using a Titerteck Multiscan ELISA plate reader with a test wavelength of 540nm and a reference wavelength of 680nm. The percentage of cellular metabolic activity was calculated using the following formula:

$$[\text{OD (sample)} - \text{OD (positive control)}] / [\text{OD (negative control)} - \text{OD (positive control)}] \times 100.$$

4.2.3.2 Propidium Iodide staining

The incorporation of propidium iodide was determined in the presence and absence of purified Stx2. PBMCs were incubated with varying concentrations of Stx2 and IPPL (Intestinal Peyer's patch Lymphocytes isolated from the ileum) populations were incubated with a fixed

concentration (2000CD₅₀) of Stx2. The freshly isolated cell populations (3x10⁵ cells/well) were incubated either 96 hours (PBMC) or 12 hours (IPPL) with Stx2 at 37°C under 5% CO₂. Following the incubation, 2.5µg/ml propidium iodide (PI) (Sigma) was added to each well and the plate was allowed to sit on ice for 20 minutes. The cells were collected and subjected to flow cytometric analyses.

4.2.3.3 Proliferation assay

Lymphocyte proliferation was determined in the presence and absence of purified Stx2. First, freshly isolated cell populations (3x10⁵ cells/well) were incubated for either 12 (IPPLs) or 96 (PBMCs) hours at 37°C under 5% CO₂ with a fixed concentration of purified Stx2 (2000CD₅₀). Twenty microliters of 0.4µCi of ³H-thymidine (Amersham Pharmacia, Piscataway, NJ) was added to each well. The plate was allowed to incubate for 6 hours at 37°C under 5% CO₂. The plates were immediately frozen until the incorporation of ³H-thymidine could be assessed in a beta counter (Topcount, PerkinElmer Inc., CA, USA). Cell proliferation was calculated as the mean counts per minute (c.p.m.) of triplicate cultures and expressed as a stimulation index (c.p.m. in the presence of stimulus / c.p.m. in the absence of stimulus).

4.2.4 Cytokine Analysis

A detailed ELISA protocol is described in section 3.6.4. After cell isolation (protocols outlined in sections 3.2.1 and 3.2.2), bovine PBMCs and IPPLs were incubated with purified Stx2 at 2000CD₅₀ for 96 and 12 hours respectively. After the appropriate incubation time, each cell type was centrifuged for 5 minutes at 14000 rpm and media was decanted from each well without disturbing the cell pellet. One hundred microliters of media was immediately applied to IFN γ , TNF α , IL12 or IL10 specific pre-coated ELISA plates and diluted in a two-fold serial dilution series for cytokine concentration evaluation.

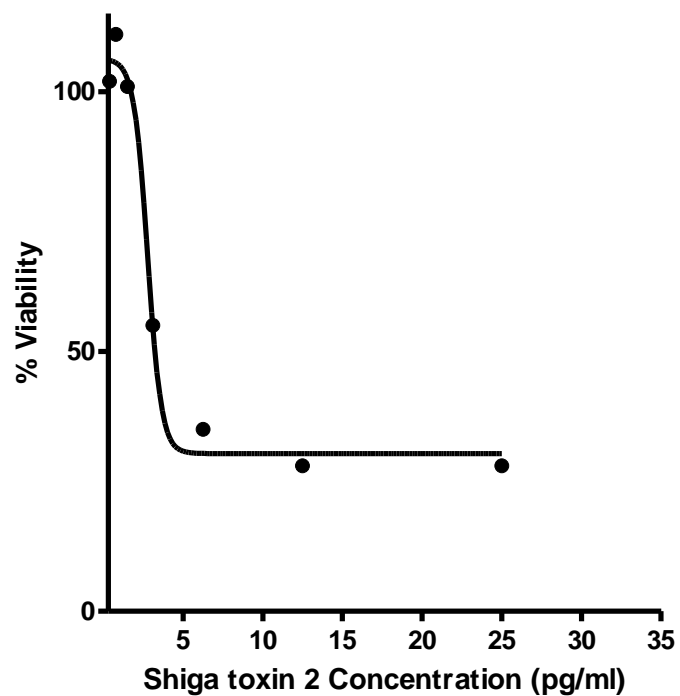


Figure 2. Cytotoxicity assay using VERO cells to determine the CD_{50} value for the Stx2 preparation. Ten-fold dilutions of the purified Stx2 preparation were added to a 96 round bottom plate and 50 μ l of VERO cell suspension (8×10^5 cells/ml) was added to each well. The toxin-cell mixture was allowed to incubate at 37°C under 5% CO_2 for 96 hours. Cell viability was assessed using MTT reduction.

4.3 Results

4.3.1 Flow Cytometric Analysis on bovine PBMC and IPPL populations to confirm expression of Shiga toxin Receptor

To confirm surface expression of the Shiga toxin receptor on bovine PBMCs and IPPLs, flow cytometric analysis using an anti-CD77/Gb₃ monoclonal antibody, was completed as described [67]. Flow cytometric analysis indicated that a percentage of both bovine PBMC and IPPL populations bound the monoclonal antibody and therefore expressed the Stx receptor on their cell surface. Approximately 7% of the IPPL isolated from the jejunum were CD77⁺ and approximately 10% of the IPPL isolated from the ileum were CD77⁺ (Figures 3 and 4). Results of PBMC staining with the anti-CD77 monoclonal antibody revealed that 13% of the population was CD77⁺. PBMC populations were also stimulated with LPS and results revealed that 22% of the stimulated bovine PBMC population was CD77⁺ (Figure 5). Therefore we can conclude that bovine lymphocyte populations isolated from the ileum, jejunum and blood express the Stx receptor on their cell surface.

4.3.2 Cellular metabolic activity of bovine PBMC and IPPL populations in the presence of purified Stx2 *in vitro*.

To determine the effect of Stx2 on cellular metabolic activity, freshly isolated bovine PBMC and IPPL populations were incubated with varying concentrations of purified Stx2 and cellular metabolic activity measured as described [68]. The results (Figure 7) indicated that Stx2 did not affect the cellular metabolic activity as assessed by MTT reduction of the bovine PBMC population *in vitro* after 4 days of incubation with the toxin. The cellular metabolic activity of bovine PBMCs incubated with either ConA or LPS after four days was approximately 100%. The cellular metabolic activity of bovine PBMCs incubated with either mitogen and purified Stx2 was also approximately 100%. Identical results were also obtained for the bovine IPPL populations which indicated that Stx2 (within the concentration range of 2ng/ml to 0.02pg/ml) did not affect their cellular metabolic activity *in vitro* as assessed by the MTT reduction assay after 12 hours of incubation with the toxin. The cellular metabolic activity of bovine IPPLs incubated with ConA or ConA + Stx2 after 12 hours was approximately 60%

(Figure 6a) and 55% (Figure 6b). Neither supplementation with ConA or LPS was required to see the effects of Stx2 on either bovine PBMC or IPPL metabolic activity.

4.3.3 Propidium Iodide staining of bovine PBMC and ileal IPPL populations after exposure to Shiga toxin 2.

The incorporation of propidium was determined in the presence and absence of purified Stx2. Propidium iodide intercalates into DNA nonspecifically and apoptotic cells expel their cellular contents into the environment including free DNA. Therefore to determine if Stx2 induces lymphocyte death, we stained bovine PBMCs and ileal IPPLs with propidium iodide after exposing them to purified Stx2 for either 96 or 12 hours (Tables 4 and 5). Approximately 60% of PI-positive IPPL cells were found in the presence of Stx2 and this was identical to the results obtained when IPPLs were incubated alone without Stx2. This was also observed in ConA-stimulated cultures. Similar results were obtained when bovine PBMCs were stained with PI. Cells were incubated either alone or stimulated with LPS and in the presence of varying concentrations of Stx2. The results indicated that similar amounts of PI was incorporated at the three toxin dilutions used when comparing stimulated cultures with or without Stx2 and nonstimulated cultures with or without Stx2. Overall, more PI was incorporated in the stimulated cultures compared to nonstimulated cultures but this is not surprising as the mitogen will induce proliferation. We therefore concluded that Stx2 does not affect lymphocyte viability within the bovine PBMC or IPPL populations *in vitro*.

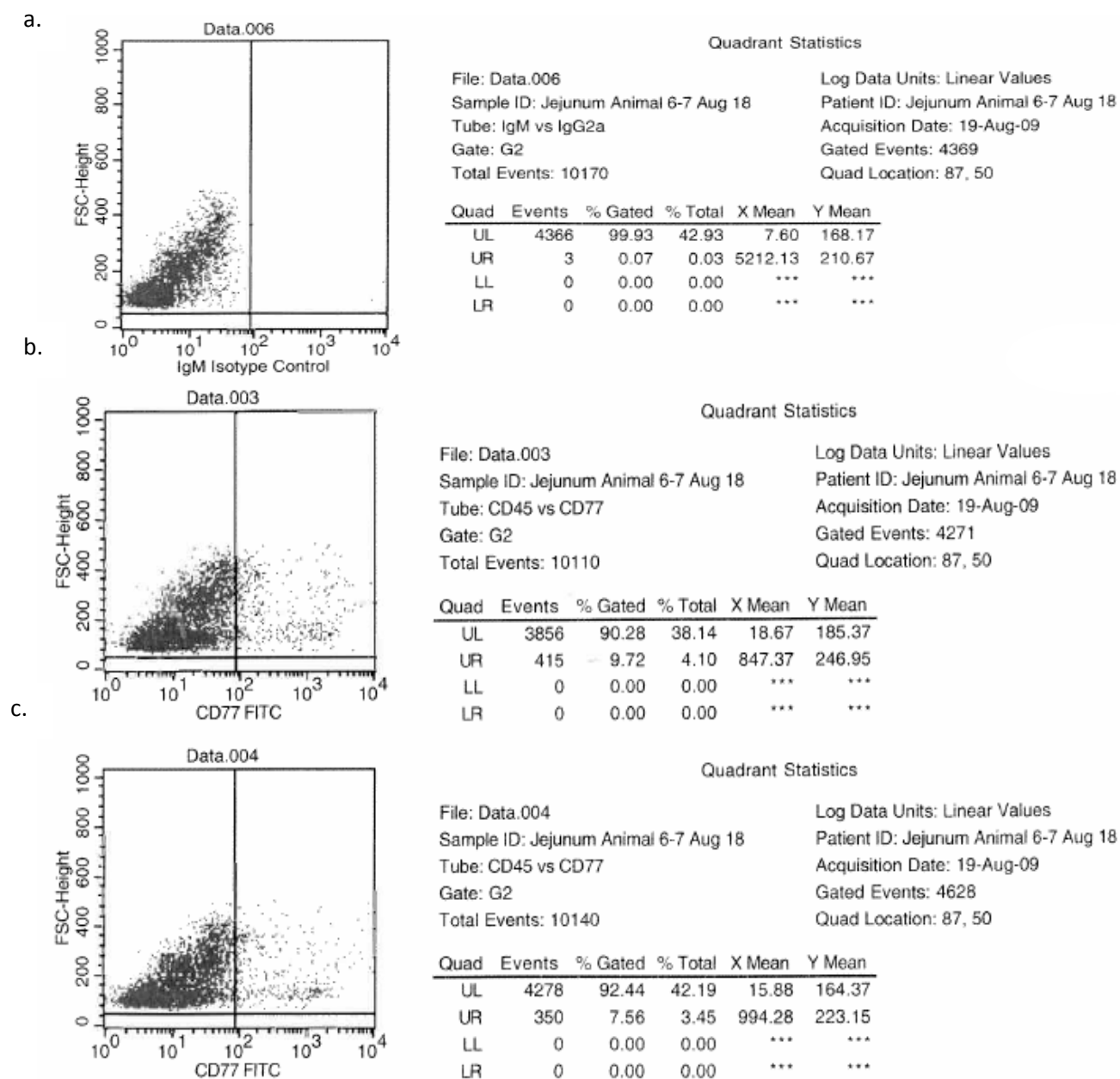


Figure 3. Flow cytometry analysis of the cells isolated from the jejunum of a six month old calf. CD45 positive cells were first identified within the population of cells that resulted from the digestion. From the resulting CD45 positive leukocytes, CD77 positive cells were identified. All samples were performed in duplicate (b) and (c). The appropriate controls (isotype and secondary antibody) (a) were included on each 96 well plate and used to exclude any non-specific antibody binding that may occur during the analysis (Table 3).

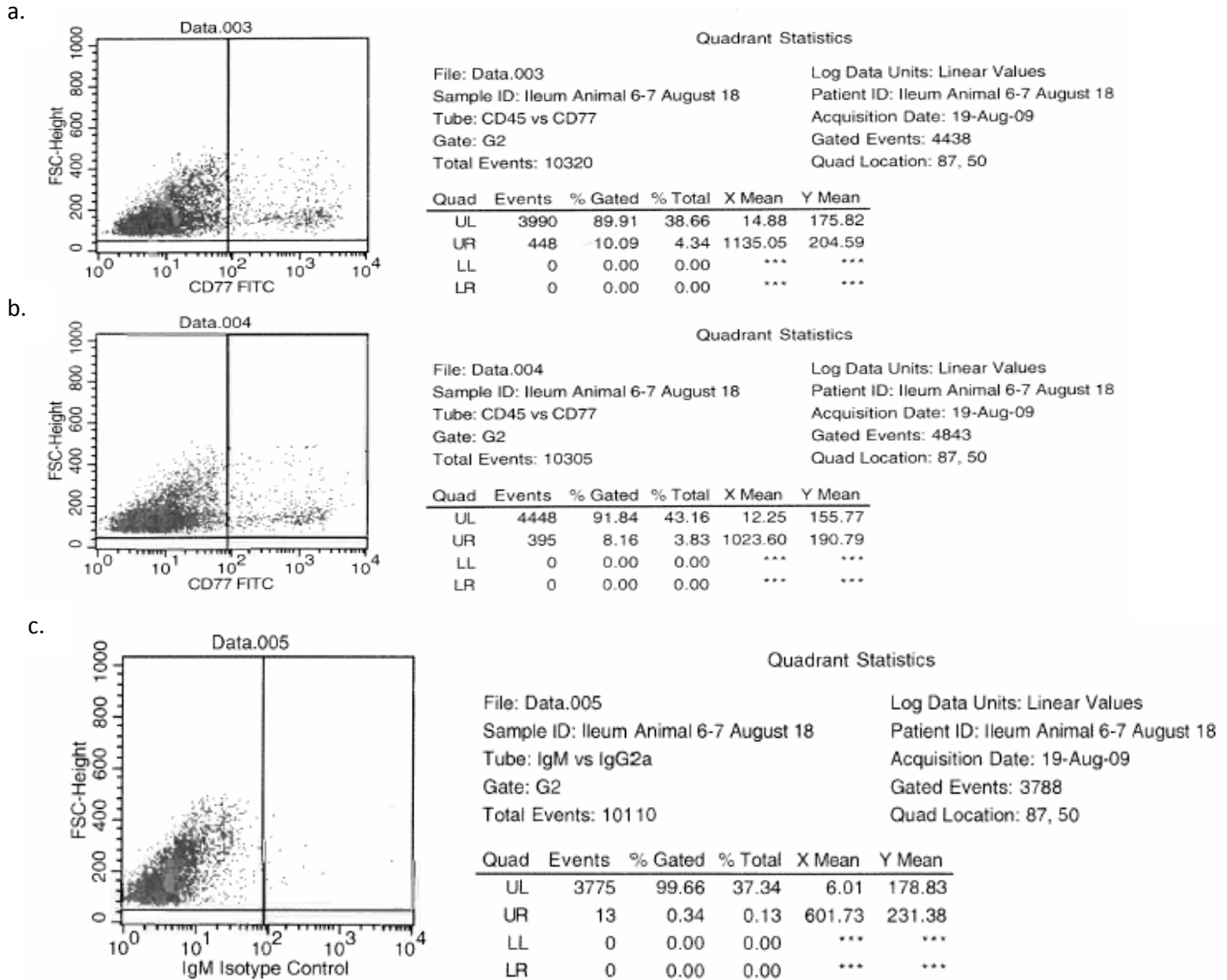


Figure 4. Flow cytometry analysis of the cells isolated from the ileum of a six month old calf. CD45 positive cells were first identified within the population of cells that resulted from the digestion. From the resulting CD45 positive leukocytes, CD77 positive cells were identified. All samples were performed in duplicate. Sample 1 (a) and sample 2 (b) each represent a 5cm² piece of the ileum taken from the same six month of calf. Ten thousand events were acquired for each sample. The appropriate controls (isotype and secondary antibody) (c) were included on each 96 well plate and used to exclude any non-specific antibody binding that may occur during the analysis (Table 3).

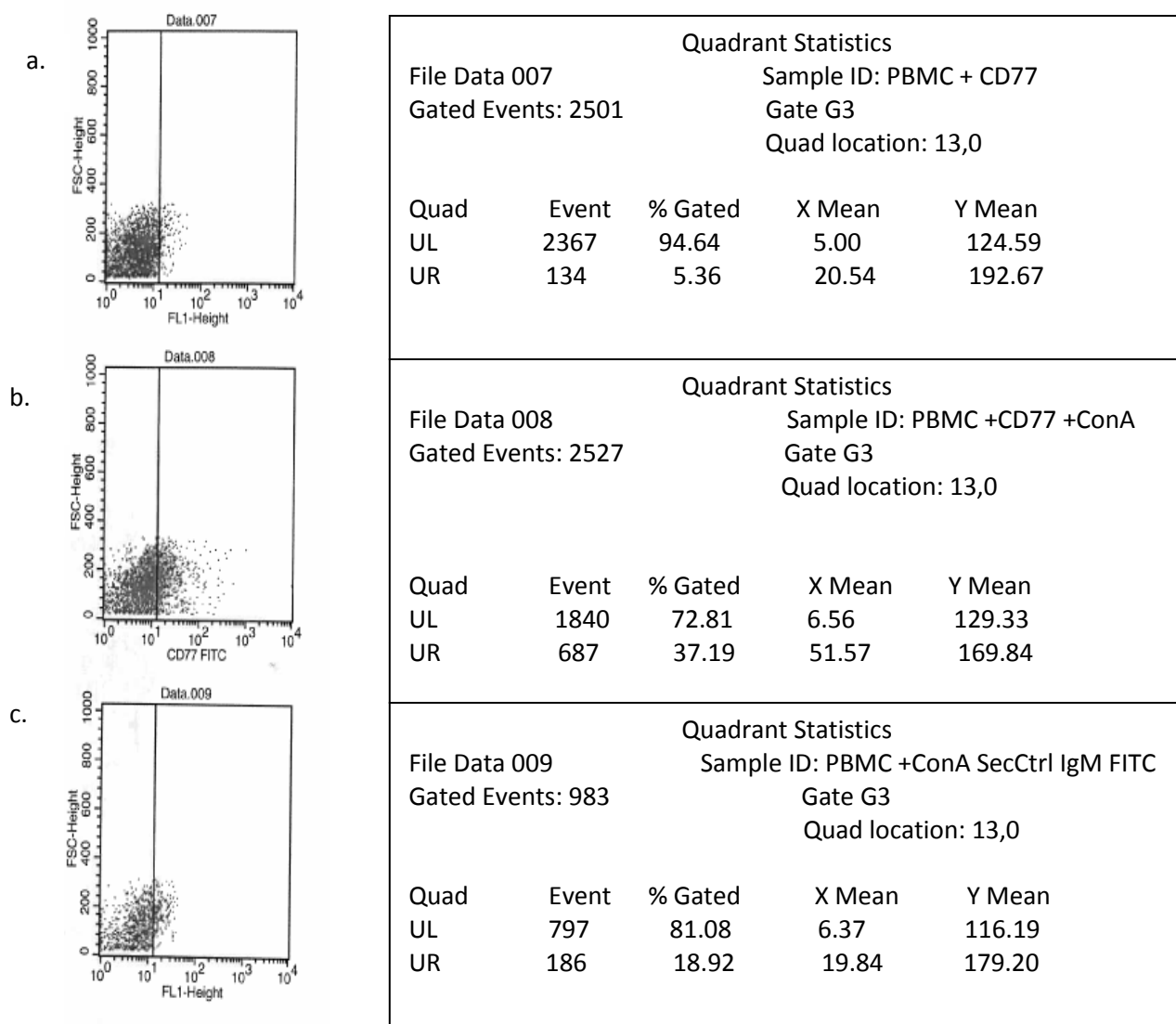


Figure 5. Flow cytometry analysis of bovine PBMC population using the anti-CD77 monoclonal antibody. Freshly isolated bovine PBMCs were subjected to flow cytometry to confirm that the cell population expresses the Stx receptor on their surface. The appropriate controls (isotype and secondary antibody) were included on each 96 well plate and used to exclude any non-specific antibody binding that may occur during the analysis (Table 3).

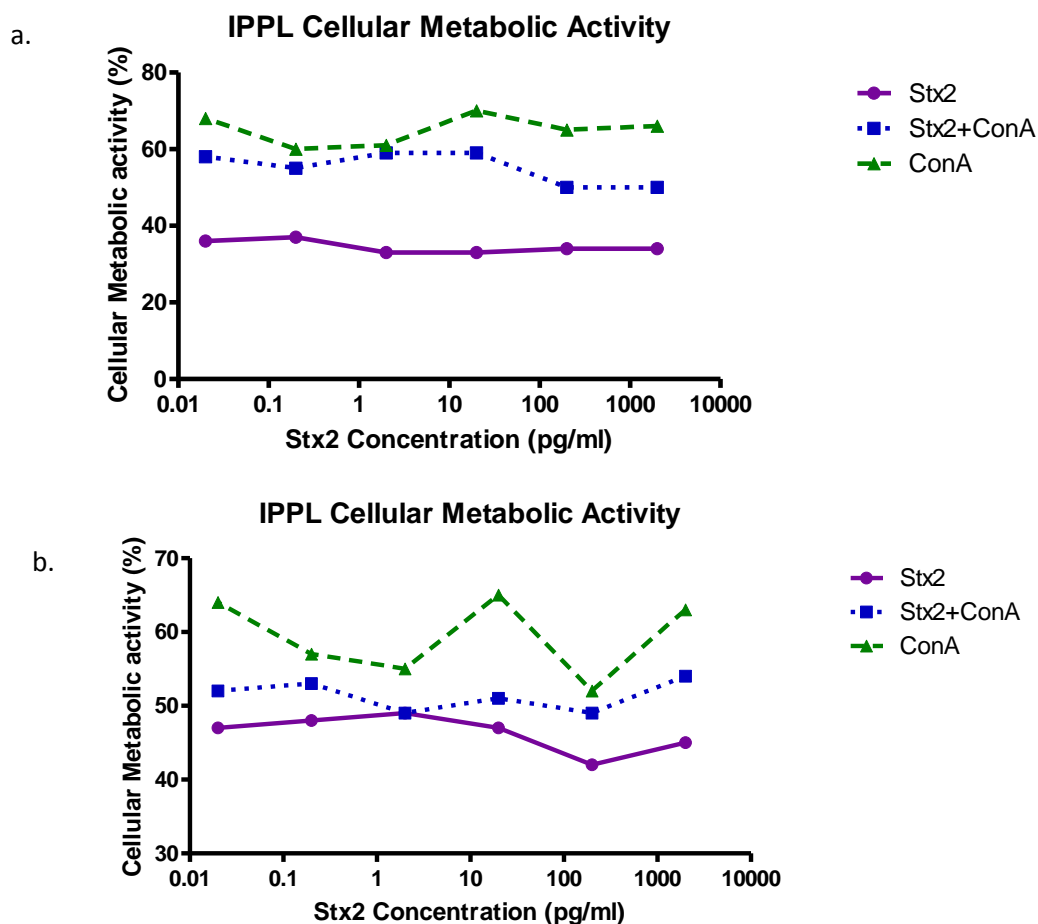


Figure 6. Effect of Stx2 on the cellular metabolic activity of bovine ileal IPPL populations *in vitro* as assessed by MTT reduction assay. Cells were incubated in a 96 well plate at 37°C under 5% CO₂ for 12 hours in the presence of a ten-fold dilution series of Stx2 [68]. The culture media was either free of mitogen or supplemented with 5µg/ml ConA. Cells incubated in media were used as a negative control and cells treated with 1% SDS were used as a positive control. Values represent the averages from triplicate determinations of one animal and ileal IPPL populations isolated from cow 1 (a) and cow 2 (b).

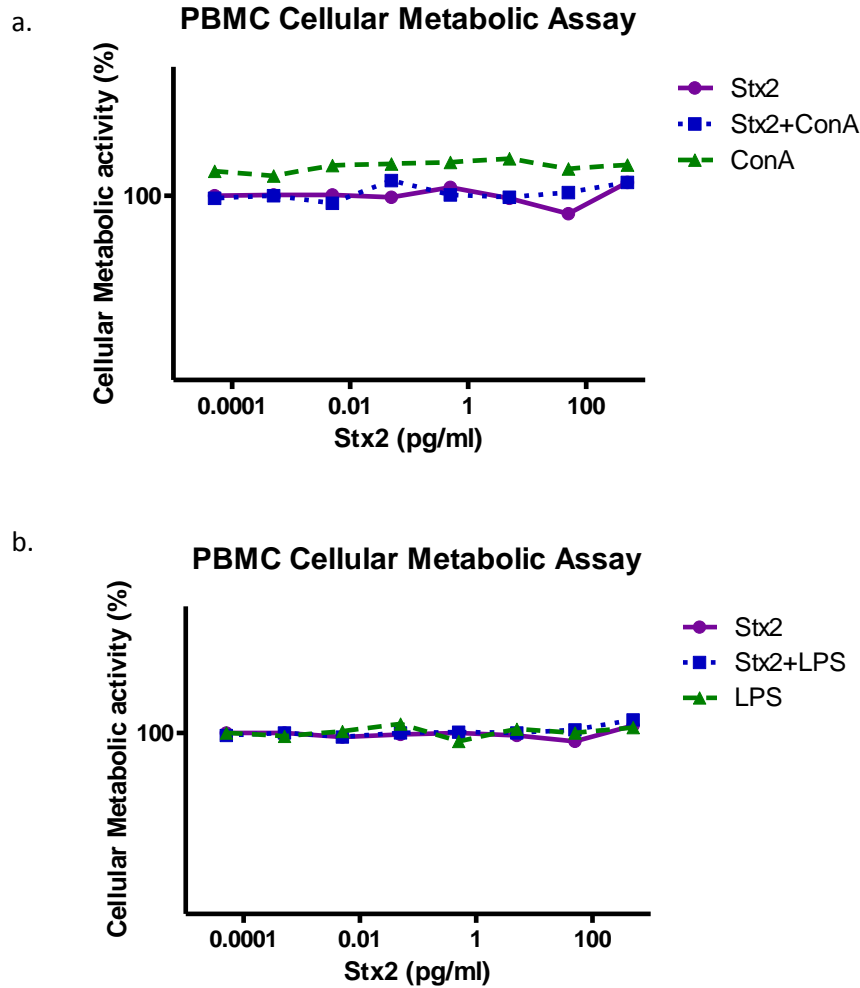


Figure 7. Effect of Stx2 on the cellular metabolic activity of bovine PBMC population *in vitro* as assessed by MTT reduction assay [183]. Cells were incubated in a 96 well plate at 37°C under 5% CO₂ for 96 hours in the presence of a ten-fold dilution series of Stx2. The culture media was either free of mitogen or supplemented with 5µg/ml ConA or 25µg/ml LPS. Cells incubated alone were used as a negative control and cells treated with 1% SDS were used as a positive control. Values represent the averages from triplicate determinations of one animal and PBMC populations isolated from cow 1 (a) and cow 2 (b).

4.3.4 Proliferation of bovine PBMC and IPPL populations exposed to Shiga toxin 2.

To determine if Stx2 inhibits proliferation in bovine lymphocytes, we assessed the incorporation of tritiated thymidine in the presence and absence of purified Stx2. A preliminary experiment was done in order to assess if results would differ if either mitogen (ConA) or toxin was added to the assay first (data not shown). The results indicated that the assay was not sensitive to either experimental condition and both mitogen and toxin were added to the assay together at time zero. The mitogen-induced proliferation of the bovine PBMC populations was not inhibited *in vitro* in the presence of Stx2 at a toxin concentration of 2000pg/ml (2000CD₅₀) [68] after 96 hours of incubation. The results indicated that the stimulation index (SI) was approximately 50 when stimulated PBMC cultures were incubated with Stx2 which was the same for stimulated PBMC cultures in the absence of Stx2 (Figure 8a). Statistical analysis using the Mann Whitney test showed proliferation of PBMC populations in the presence of Stx2 were significantly lower ($P<0.01$) than either PBMC population stimulated with ConA (in the presence or absence of Stx2) (Figure 8a).

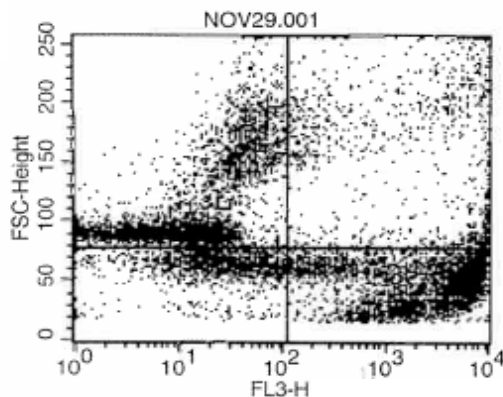
Proliferative responses of the bovine ileal IPPL populations were measured using cells from four different calves. The calves were approximately three months of age and ConA was chosen as the positive control. The results in Figure 8b indicate that Stx2 inhibited the mitogen-induced proliferation of the ileal IPPL population *in vitro*. When stimulated IPPL cultures were incubated with Stx2, the SI was approximately 3.5. The results of stimulated IPPL cultures in the absence of Stx2 showed a SI of 13. Statistical analysis using the Mann Whitney showed that they were significantly different ($P<0.05$), indicating that Stx2 was able to downregulate bovine immune responses by inhibiting the mitogen-induced proliferation of the ileal IPPL populations *in vitro*. The mitogen, Con A was also able to stimulate proliferation in the ileal IPPL populations that differed significantly ($P<0.01$) from the unstimulated cultures. The SI values determined for the IPPL populations were very low suggesting a percentage of the population after 12 hours is no longer alive (Figure 8b).

4.3.5 Cytokine concentration determination in the presence of Shiga toxin 2.

To determine if Stx2 modulates bovine immune responses through cytokine production, bovine PBMC and ileal IPPL lymphocyte populations were incubated with purified Stx2 at a

concentration of 200CD₅₀ for varying time intervals. After incubation with Stx2, culture media was tested for the presence of different cytokines. Unstimulated cells incubated without Stx2 were compared to unstimulated cells incubated with the toxin and cells stimulated with the mitogen ConA were compared to cells stimulated with ConA and purified Stx2 (Figures 9 and 10). Statistical analysis using the Mann-Whitney test showed that ConA-stimulated PBMC and IPPL populations produced significantly higher ($P<0.05$) IL4, TNF α , IFN γ and IL10 concentrations than unstimulated cultures (for example PBMC/IPPL + ConA versus PBMC/IPPL and PBMC/IPPL + ConA +Stx2 versus PBMC/IPPL + Stx2). The results indicate that Stx2 did not significantly alter either IL12 (PBMC), IL4 (IPPL), TNF α or IL10 levels in ConA-stimulated bovine PBMC and IPPL preparations (PBMC/IPPL + ConA versus PBMC/IPPL + ConA+ Stx2). IFN γ concentrations produced by PBMCs stimulated with ConA were significantly higher ($P<0.05$) than IFN γ concentrations produced by ConA-stimulated PBMCs in the presence of Stx2. This result was not observed with the IPPL population. IL12 concentrations produced by unstimulated PBMCs were significantly lower ($P<0.05$) than IL12 concentrations produced by ConA-stimulated PBMCs as well significantly lower ($P<0.05$) IL12 concentrations were observed upon comparing unstimulated PBMCs and unstimulated PBMCs incubated with Stx2.

a.



File: NOV29.001

Sample ID: CELLS ONLY NO TREATMENT

Acquisition Date: 29-Nov-06

Gated Events: 10000

X Parameter: FL3-H (Log)

Quad Location: 107, 77

Log Data Units: Linear Values

Patient ID:

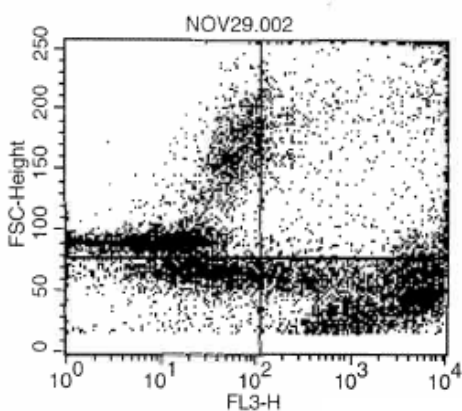
Gate: No Gate

Total Events: 10000

Y Parameter: FSC-H FSC-H

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	3939	39.39	39.39	21.93	112.01
UR	944	9.44	9.44	3858.76	159.34
LL	1446	14.46	14.46	32.43	62.21
LR	3671	36.71	36.71	3883.43	48.60

b.



Quadrant Statistics

File: NOV29.002

Sample ID: PBMC +TOXIN 0 DILN

Acquisition Date: 29-Nov-06

Gated Events: 10000

X Parameter: FL3-H (Log)

Quad Location: 107, 77

Log Data Units: Linear Values

Patient ID:

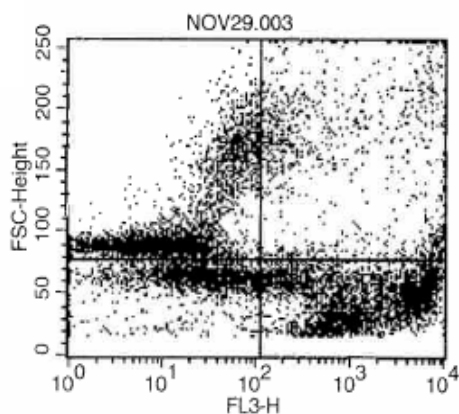
Gate: No Gate

Total Events: 10000

Y Parameter: FSC-H FSC-Height (Lin)

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	3463	34.63	34.63	25.85	111.47
UR	1150	11.50	11.50	3808.69	164.68
LL	1908	19.08	19.08	31.54	61.61
LR	3479	34.79	34.79	3105.33	48.62

c.



Quadrant Statistics

File: NOV29.003

Sample ID: PBMC +TOXIN 1 DILN

Acquisition Date: 29-Nov-06

Gated Events: 10000

X Parameter: FL3-H (Log)

Quad Location: 107, 77

Log Data Units: Linear Values

Patient ID:

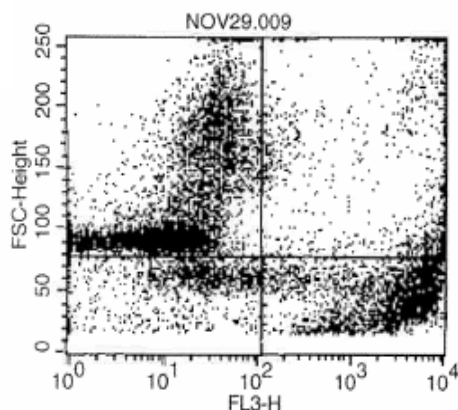
Gate: No Gate

Total Events: 10000

Y Parameter: FSC-H FSC-Height (Lin)

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	3553	35.53	35.53	24.34	108.67
UR	1035	10.35	10.35	2886.26	163.37
LL	1903	19.03	19.03	32.60	61.48
LR	3509	35.09	35.09	2337.47	44.72

d.



File: NOV29.009

Sample ID: PBMC +TOXIN+LPS 10-0 DILN

Acquisition Date: 29-Nov-06

Gated Events: 10000

X Parameter: FL3-H (Log)

Quad Location: 107, 77

Log Data Units: Linear Value

Patient ID:

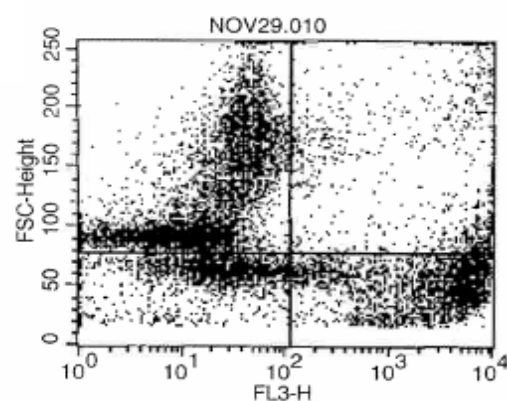
Gate: No Gate

Total Events: 10000

Y Parameter: FSC-H FSC-H

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	5284	52.84	52.84	22.17	121.77
UR	931	9.31	9.31	4838.68	147.48
LL	1137	11.37	11.37	30.01	55.93
LR	2648	26.48	26.48	3741.46	46.14

e.



Quadrant Statistics

File: NOV29.010

Sample ID: PBMC +TOXIN+LPS 10-1 DILN

Acquisition Date: 29-Nov-06

Gated Events: 10000

X Parameter: FL3-H (Log)

Quad Location: 107, 77

Log Data Units: Linear Value

Patient ID:

Gate: No Gate

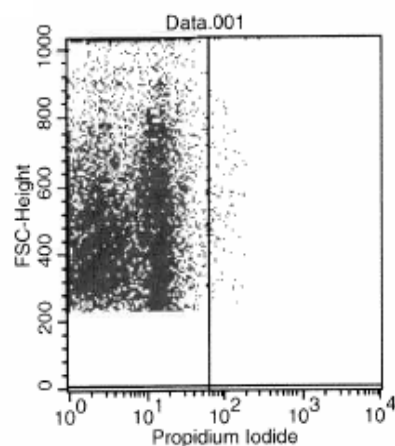
Total Events: 10000

Y Parameter: FSC-H FSC-H

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	5163	51.63	51.63	23.99	124.10
UR	809	8.09	8.09	4119.61	149.16
LL	1726	17.26	17.26	29.42	59.29
LR	2302	23.02	23.02	3574.81	49.65

Table 4. Survival of Stimulated and Unstimulated Bovine PBMCs after Exposure to Purified Stx2. Propidium iodide staining of bovine PBMCs after incubation with a) PBMCs only b) PBMCs + 2000CD₅₀ purified Stx2 c) PBMCs + 200CD₅₀ d) PBMCs + 0.25 µg/ml LPS + 2000CD₅₀ e) PBMCs + 0.25 µg/ml LPS + 200CD₅₀

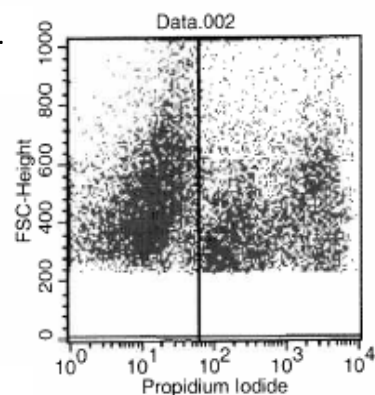
a.



File: Data.001 Sample ID: LPL Cell Control
 Patient ID: Calf Acquisition Date: 26-Aug-08
 Gate: G1 Gated Events: 10680
 Quad Location: 63, 7

Quad	Events	% Gated	X Mean	Y Mean
UL	10569	98.96	9.08	489.52
UR	111	1.04	100.48	555.23

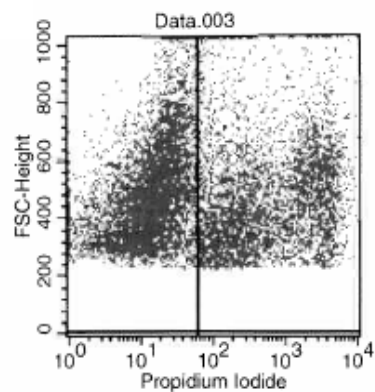
b.



File: Data.002 Sample ID: LPL Cell Control PI stair
 Patient ID: Calf Acquisition Date: 26-Aug-08
 Gate: G1 Gated Events: 10157
 Quad Location: 63, 7

Quad	Events	% Gated	X Mean	Y Mean
UL	5897	58.06	17.24	491.81
UR	4260	41.94	1363.26	463.46

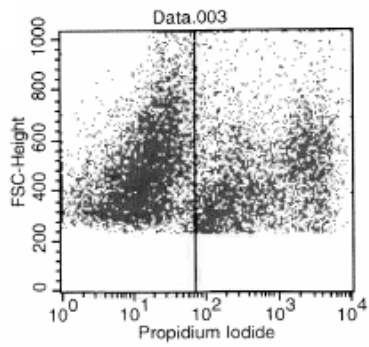
c.



File: Data.003 Sample ID: LPL plus STX2
 Patient ID: Calf Acquisition Date: 26-Aug-08
 Gate: G1 Gated Events: 10299
 Quad Location: 63, 7

Quad	Events	% Gated	X Mean	Y Mean
UL	5966	57.93	18.58	488.36
UR	4333	42.07	1365.70	471.89

d.

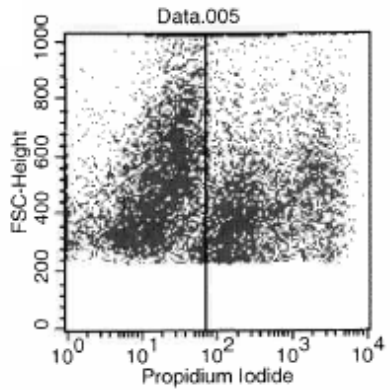


File: Data.003
Patient ID: Call 2
Gate: G1
Quad Location: 70, 0

Sample ID: LPL plus STX2
Acquisition Date: 26-Aug-08
Gated Events: 10299

Quad	Events	% Gated	X Mean	Y Mean
UL	6078	59.02	19.47	488.66
UR	4221	40.98	1400.15	471.03

e.

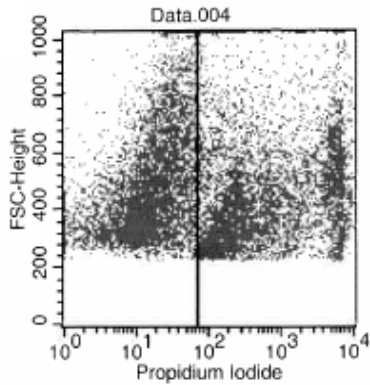


File: Data.005
Patient ID: Calf
Gate: G1
Quad Location: 70, 0

Sample ID: LPL Con A
Acquisition Date: 26-Aug-08
Gated Events: 10228

Quad	Events	% Gated	X Mean	Y Mean
UL	5739	56.11	21.81	504.66
UR	4489	43.89	919.24	475.06

f.



File: Data.004
Patient ID: Calf
Gate: G1
Quad Location: 70, 0

Sample ID: LPL Con A plus Stx2
Acquisition Date: 26-Aug-08
Gated Events: 10508

Quad	Events	% Gated	X Mean	Y Mean
UL	5337	50.79	20.98	492.54
UR	5171	49.21	1842.08	475.80

g.

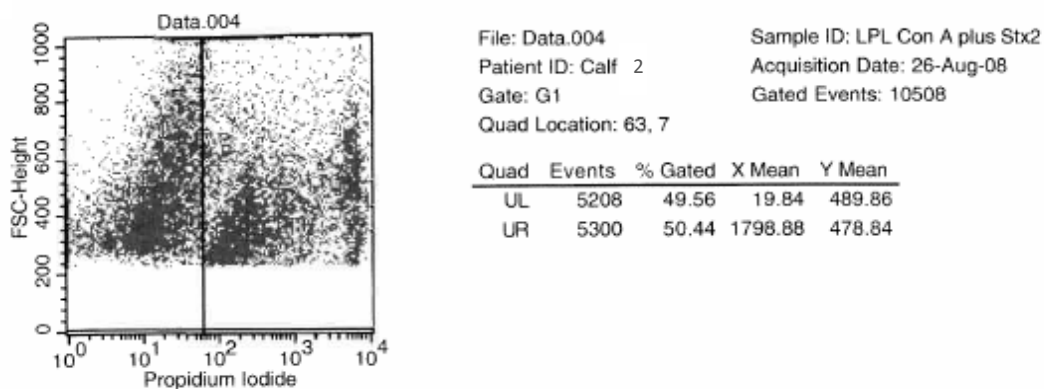


Table 5. Survival of Stimulated and Unstimulated Bovine ileal IPPLs after Exposure to Purified Stx2. Propidium iodide staining of bovine lymphocytes isolated from the ileal Peyer's Patches incubated with a) IPPL alone b) IPPL alone (PI control) c) IPPL + 2000CD₅₀ purified Stx2 (cow 1) d) IPPL + 2000CD₅₀ purified Stx2 (cow 2) e) IPPL + 5µg/ml ConA (cow1) f) IPPL + 5µg/ml ConA + 2000CD₅₀ purified Stx2 (cow 1) f) IPPL + 5µg/ml ConA (cow1) g) IPPL + 5µg/ml ConA + 2000CD₅₀ purified Stx2 (cow 2).

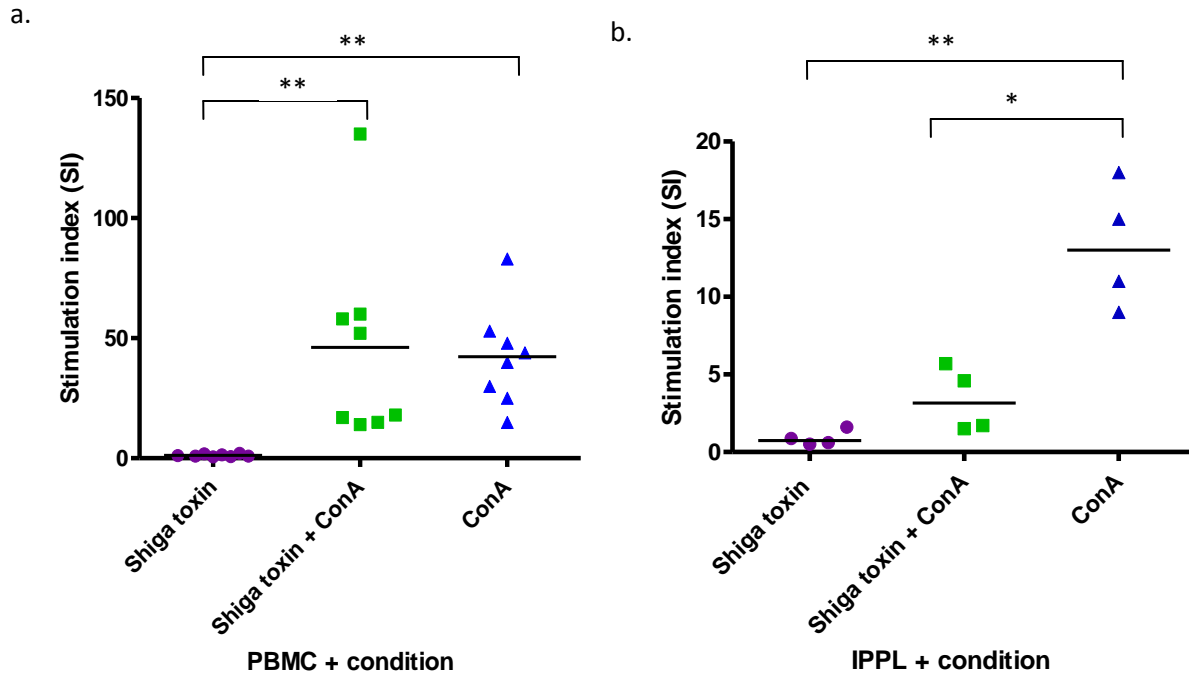


Figure 8. Proliferation of bovine PBMC and IPPL preparations in the presence and absence of purified Stx2. Freshly isolated a) PBMCs (2.5×10^5) and b) IPPLs (2.5×10^5) + ConA ($5 \mu\text{g/ml}$) + Stx2 (2000CD_{50}) were incubated at 37°C at 5% CO_2 . After 96 hours of incubation (12 hours for IPPLs), the incorporation of ^3H -thymidine was assessed using a beta counter as described above. * $P < 0.05$; ** $P < 0.01$.

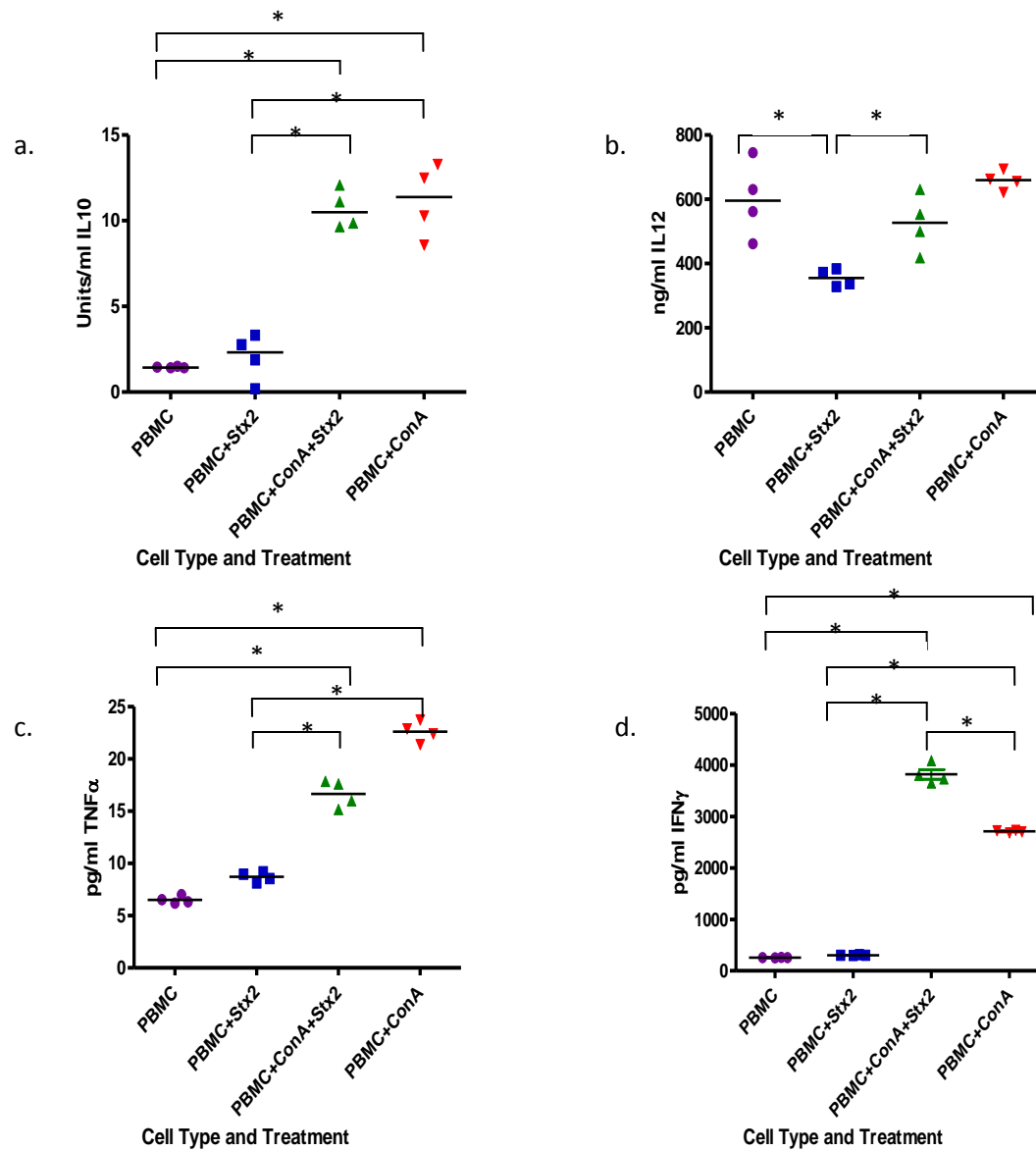


Figure 9. Cytokine production (IL10, IL12, TNF α and IFN γ) by bovine PBMCs in the presence of Stx2. Freshly isolated bovine PBMC preparations were either stimulated with ConA (5 μ g/ml) and incubated with 2000CD₅₀ Stx2 (■, ▲) or remained unstimulated and incubated with 2000CD₅₀ Stx2 (●, ▼). Cytokine production including a) IL10, b) IL12, c) TNF α and d) IFN γ was determined using cytokine-specific capture ELISAs. Statistical analysis was done using the Mann Whitney test. *P<0.05.

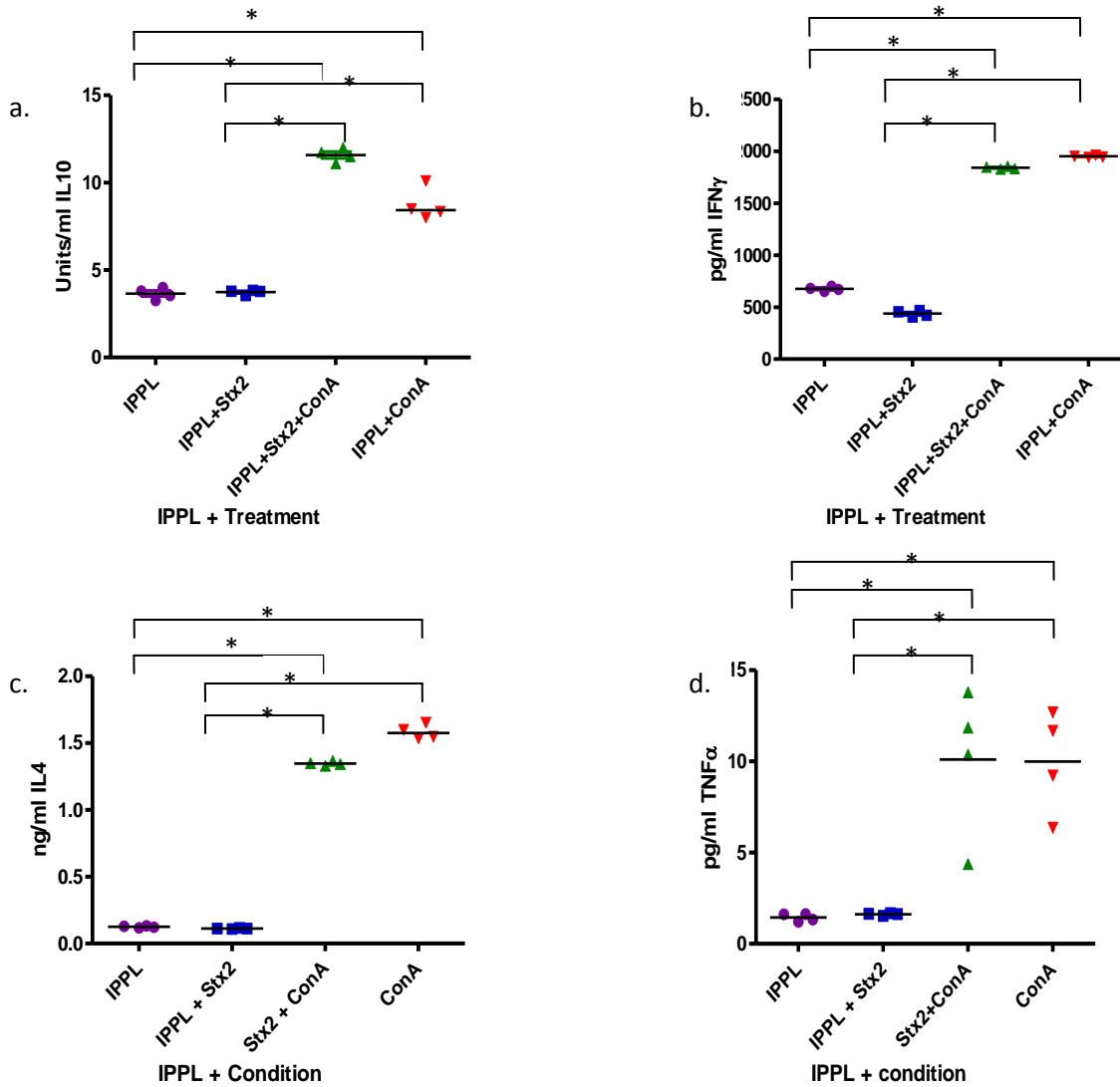


Figure 10. Cytokine production (IL10, IFN γ , IL4, and TNF α) by bovine IPPLs in the presence of Stx2. Freshly isolated bovine ileal IPPL preparations were either stimulated with ConA (5 μ g/ml) and incubated with 2000CD₅₀ Stx2 (▲, ▼) or remained unstimulated and incubated with 2000CD₅₀ Stx2 (●, ■). Cytokine production including a) IL10, b) IFN γ , c) IL4 and d) TNF α was determined using cytokine-specific capture ELISAs. Statistical analysis was done using the Mann Whitney test. *P<0.05.

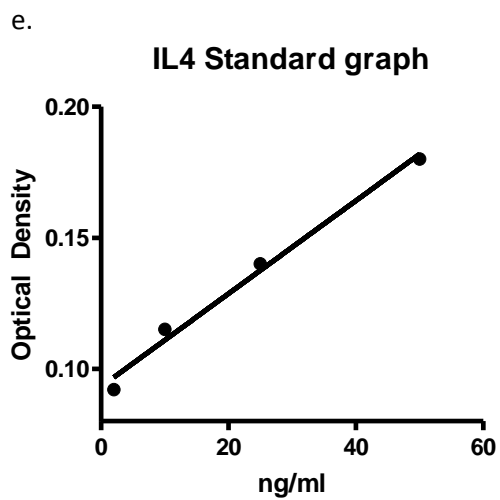
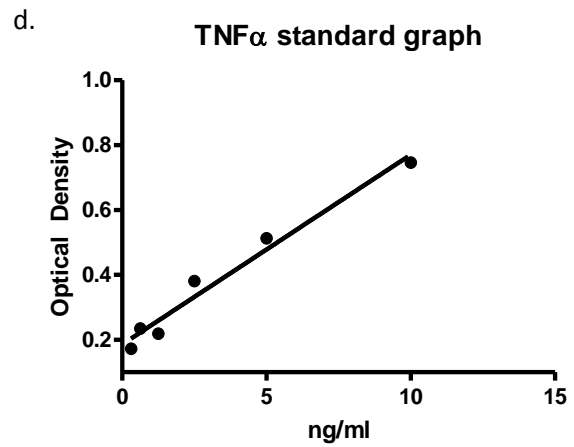
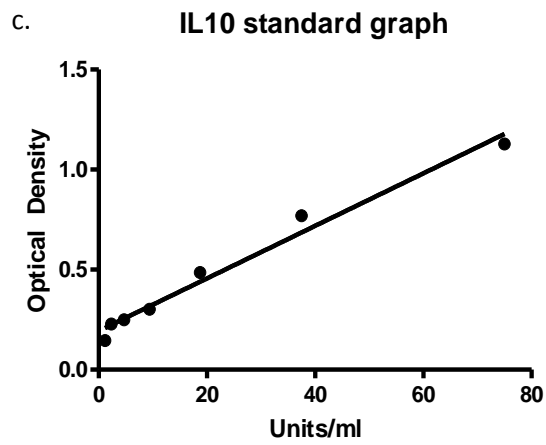
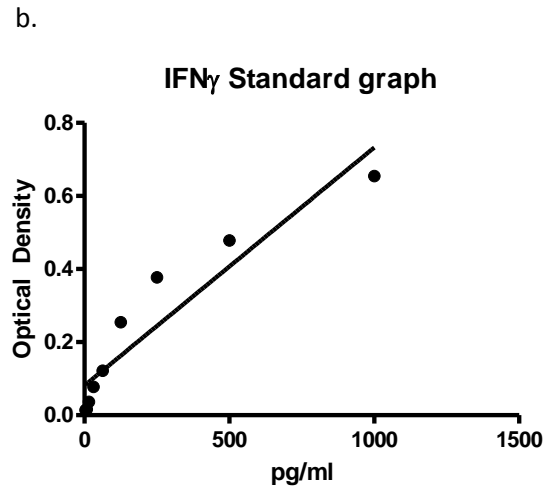
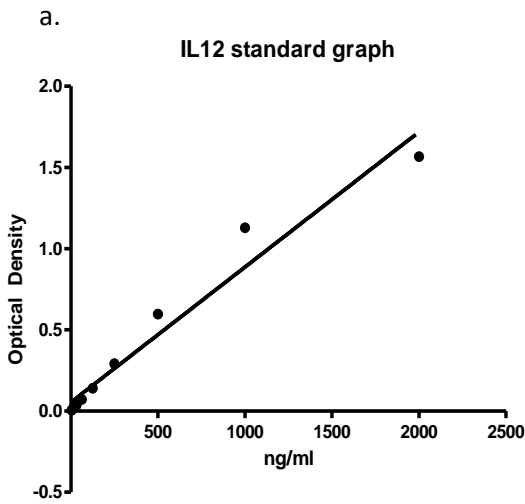


Figure 11. Cytokine standard graphs. Standard graphs were produced using a) rHuIL12, b) rBoIFN γ , c) rBoIL10, d) rBoTNF α and e) rBoIL4 in order to determine the cytokine concentrations resulting from the *in vitro* incubation of bovine PBMCs and IPPLs in the presence of Stx2 (Table 2). Two-fold dilutions of each cytokine standard were applied to a 96 round bottom plate and the optical densities were recorded and graphed.

4.4 Discussion

To date, no clinical disease or histopathology has been reported in cattle following infection with STEC. Experimentally infected calves less than three weeks of age develop diarrhea but these symptoms have been attributed primarily to Stx1 secreting STEC strains [184]. The purpose of this study was to investigate the effect of Stx2 on bovine immune cells. Previously, Dean-Nystrom et al [70] showed that the ileum and colon of experimentally infected calves suffering from STEC-induced diarrhea were colonized with viable bacteria thus confirming the presence of STEC within the bovine gut. In 2002, Stamm et al [178] showed that lymphocytes isolated from the bovine gut as well as from blood express a functional Stx receptor on their surface confirming that the shiga toxins can interact with cells of the immune system within the bovine host. This led us to hypothesize that Shiga toxin 2 could modulate the bovine mucosal immune response thereby contributing to STEC pathogenesis. This hypothesis is supported by two previous observations, including one by Menge et al [167] who reported a reduced number of intraepithelial lymphocytes after exposure to Stx1 and as well by Smith et al [171] who also reported a depletion of lymphocyte numbers in the submucosal lymphoid follicles in two five week old calves infected with an STEC strain.

In the present study we showed that Shiga toxin 2 inhibited the mitogen-induced proliferation of lymphocytes *in vitro* originating from the Peyer's patches of the ileum. This inhibition was seen after 12 hours of incubation with purified Stx2 at a concentration of 2000CD₅₀ (approximately 2000pg/ml). This effect was seen using four individual ileal IPPL preparations originating from four 3-month old Holstein calves (Figure 8b). Mangeney et al [185] have previously demonstrated a correlation between Gb₃ expression and cellular activation/differentiation. We have shown in this study that unstimulated IPPL express the Gb₃ receptor on their surface although at low levels. Therefore a mitogen-induced increase in Gb₃ expression may account for the activation-dependent effect of Stx2. Cellular metabolic activity of the same ileal IPPL populations remained unaltered in the presence of 2000CD₅₀ Stx2. This result agreed with the PI staining of the same population of cells (Table 3). Although it has been shown that Stx1 induces apoptosis in VERO cells after 8 hours of incubation as well as the bovine lymphoma cell line BL-3 and the human B lymphoma cell line Daudi [68, 186, 187], we did not observe a decrease in lymphocyte viability due to Stx2 after 12 hours of incubation at

2000CD₅₀. The perturbation of cytokines by Stx2 was also examined to fully understand the immunomodulating effects of Stx2. Menge et al [69] showed that incubation with Stx1 increased the percentage of bovine PBMCs expressing IL-2 receptor and a reduction in CD71-expressing lymphocytes suggesting Stx1 may inhibit cell activation by inhibiting IL2 release. In the present study, we examined cytokine profiles of IL10, IL4, TNF α and IFN γ after incubation with Stx2 after 12 hours. Results indicated no significant differences in cytokine concentrations when stimulated ileal PPLs were either in the presence or absence of Stx2.

To fully examine the modulatory role by Stx2 on bovine immune cells, we determined the effect of Stx2 on bovine PBMCs *in vitro* as well. Flow cytometric analysis using a monoclonal antibody to the receptor Gb₃ indicated that bovine PBMCs do express the shiga toxin receptor on their surface (Figure 4 and 5). Confirmation of the binding specificity of the monoclonal antibody to CD77 was done by Menge et al in 2001 [67]. Flow cytometry result indicated that circulating lymphocytes within the bovine host could interact with Stx2 which is in agreement with results obtained by Menge et al 2001 [67]. Cellular metabolic activity was determined after 96 hours of incubation with 2000CD₅₀ Stx2 and results indicated that Stx2 does not alter cellular metabolic activity *in vitro* (Figure 7). This was in agreement with the PI staining done on the same population of cells. The results indicated no increase in PI-positive PBMCs after exposure to purified Stx2 for 4 days (Table 3). This was in agreement with results observed by Menge et al [68] in 1999 when they concluded that Stx1 does not affect bovine PBMC viability after 96 hours of incubation. The perturbation of cytokines in the presence of Stx2 was also examined. The results of our cytokine specific ELISAs indicated that Stx2 does not modulate IL10, TNF α or IL12 cytokine concentrations when comparing ConA-stimulated cultures either in the presence or absence of purified Stx2. This result agrees with Menge et al [69] when they concluded that Stx1 affects bovine lymphocytes independent of various cytokines including TNF α . IFN γ concentrations in ConA-stimulated PBMC cultures though did differ significantly. ConA-stimulated PBMCs in the presence of Stx2 produced more IFN γ (approximately 4000pg/ml) than ConA-stimulated PBMCs which produced approximately 3000pg/ml.

Dean-Nystrom et al [70] showed that calves experimentally inoculated with *E. coli* O157:H7 strain 933 showed bacterial colonization in their rectum, cecum, colon and ileum and

Nart et al [17] concluded from their studies that *E. coli* O157:H7 colonizes the terminal rectum demonstrating tissue tropism for *E. coli* O157:H7 within the bovine host. In 1995 Cray et al [188] concluded from their study using preweaned calves and adult cattle that *E. coli* O157:H7 does not spread from the alimentary tract to other organs such as the kidneys, liver or spleen. Therefore, we felt it was logical to study the effect of Stx2 on the population of lymphocytes exposed to *E. coli* O157:H7. In young ruminants, the ileum is composed primarily of Peyer's patch tissue consisting of contiguous aggregations of lymphoid follicles [137]. In the present study, we isolated lymphocyte populations from the Peyer's patches and results indicate that Stx2 downregulates lymphocyte proliferation in these specialized immune structures. Magnuson et al [189] showed that intestinal epithelial cell proliferation was lower in cattle that remained culture positive for *E. coli* O157:H7 than intestinal epithelial cell proliferation in cattle that cleared the bacteria. In 2006, Hoffman et al [190] reported that cattle inoculated with an Stx negative O157 strain developed significant PBMC proliferation to an Stx2⁺ heat-killed strain after being challenged with an Stx2⁺ O157 strain compared to the group of cattle initially inoculated with an Stx2⁺ strain. These results, along with the current results all suggest that Shiga toxins play a role in STEC pathogenesis in cattle. Current studies failed to show a relationship between the induction of attaching and effacing proteins such as intimin and persistent infection of *E. coli* O157:H7 suggesting that an alternative virulence factor may be responsible [191, 192]. Cattle shed *E. coli* O157:H7 intermittently throughout their lifetime with studies revealing up to 75% of dairy cattle and 63% of feedlots having culture-positive animals [193]. An individual animal can remain culture positive for over one year but usually averages 30 days with no seasonal preference [194, 195]. Recent reviews by Meyer-Broseta et al [196, 197] concluded that *E. coli* O157:H7 is "ubiquitously distributed within cattle" which remain clinically normal. Therefore it is no surprise that the relationship between cattle and *E. coli* O157:H7 needs to be understood in order to reduce colonization in cattle and subsequently lower exposure to the human population. This study provides one piece of evidence that supports the immunomodulating capabilities of Shiga toxins particularly Stx2 within the bovine host. We speculate that the percentage of receptor-bound Stx2 that is not internalized perturbs normal lymphocyte functions, including proliferation, thereby drastically reducing the magnitude of the bovine immunological response.

5.0 Effect of Shiga Toxin 2 on Cytokine Secretion by Bovine Intestinal Epithelial Cells

5.1 Introduction

Upon infection with STEC, humans may develop a series of clinical symptoms that range in severity; watery diarrhea develops into HC involving bloody diarrhea, abdominal pain and fever (70% of cases) [38]. If infection persists, HUS may develop within six days of infection and include symptoms such as abdominal pain, vomiting, bleeding from the nose and decreased urine output which ultimately may lead to acute kidney failure. The mortality rate for HUS is 5-10% [9, 81]. Thus, following major human outbreaks, STEC have been considered an emerging zoonotic pathogen of significant public health concern.

Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) produced by STEC are one group of principle virulence factors attributed to the progression of disease in humans. Shiga toxin 2 is an A-B protein comprised of one A subunit to every five B subunits. The B subunits are responsible for recognizing and binding to the receptor, Gb₃. Traditionally the activity of the Shiga toxins is cytotoxic. The A subunit of the toxin is internalized via clathrin-coated pits where it is responsible for inhibiting protein synthesis [52].

Studies on Shiga toxins and cytokine induction/inhibition have proven difficult with variable results due to species-specific and cell type-specific differences. In humans, data has suggested that Shiga toxins and cytokines work cooperatively to create the pathological changes observed during HUS [52]. Pro-inflammatory cytokines such as TNF, IL6 and IL8 have been shown to be elevated in the urine but not in the serum of HUS patients suggesting a renal source of production [198]. In cattle, however, Stxs do not appear to behave as proinflammatory factors as they do in humans. Moussay et al [199] showed using bovine intraepithelial lymphocytes that Stx1 did not alter IL2, TNF α or IFN γ mRNA transcript levels. As well Menge et al [69] concluded that Stx1 did not modulate bovine immune responses through a cytokine-mediated effect. Finally, using a bovine ligated loop model, Stevens et al [200] revealed that Stx1 does not induce intestinal inflammation *in vivo* therefore suggesting that Stxs are involved in STEC colonization and pathogenesis.

To date, the interaction between human or bovine intestinal epithelial cells and the Shiga toxins remains obscure. Because STEC is a non-invasive pathogen, it has been hypothesized that

the Stxs cross the human intestinal epithelium, to reach the bloodstream and travel to susceptible organs. Therefore, the intestinal epithelium is one of the first lines of defence and how it responds to these invading toxins has different implications for human and cattle hosts. The production of chemokines by intestinal epithelial cells directs other immune cells in the adjacent and underlying mucosa ultimately affecting the generation of an immune response. Recently, Thorpe et al showed that Stx1 significantly induces C-X-C chemokines such as IL8, GRO- α and GRO- β at the mRNA level in a human intestinal epithelial cell line HCT-8 suggesting that in humans they have an important role in providing chemoattractant signals in response to infection [201, 202]. The generation of chemokines is important for neutrophil recruitment which is part of the acute inflammation during haemorrhagic colitis in humans. Experiments using bovine intestinal epithelial cell lines though showed Stx1 was unable to stimulate the release of granulocyte chemoattractants *in vitro* or induce intestinal inflammation *in vivo* [199, 200].

In the present study, we hypothesize that Stx2 modulates the local bovine immune response within the gastrointestinal tract by downregulating cytokine gene expression from intestinal epithelial cells. We therefore tested the effect of purified Stx2 and a secreted protein preparation isolated from a wild type Stx2⁺ *E. coli* strain on the expression of selected cytokine genes *in vitro* by real-time PCR using a bovine intestinal epithelial cell line. Recent publications in support of our hypothesis have shown that Stx1 inhibits the activation and proliferation of bovine lymphocytes *in vitro* [68]. Published data (Chapter 4) from our laboratory suggests that purified Stx2 inhibits the mitogen-induced proliferation of lymphocytes *in vitro* isolated from the ileal Peyer's patches of cattle. In 2004, Menge et al [203] showed that Stx1 depletes the ileal mucosa of CD8⁺ T cells in a ligated loop model. As well published data (Chapter 6) from our laboratory suggests Stx2 depletes the ileal Peyer's patches of CD4⁺ T cells during a ligated loop model of STEC infection. Finally, Moussay et al [199] recently showed that Stx1 failed to alter cytokine-specific gene expression of multiple cytokines from intraepithelial lymphocytes isolated from the ileum of cattle. Consequently the purpose of this study was to examine if Stx2 alters transcription of selected cytokines genes. Therefore, these results will supplement our knowledge as to how STEC controls intestinal inflammation and the mucosal immune defence in cattle consequently permitting colonization and persistence.

5.2 Materials and Methods

5.2.1 Culturing of bovine intestinal epithelial cell line

The bovine intestinal epithelial cell line used in the present study has been previously characterized and described by Kauschik et al [204] and has been confirmed to be suitable for studying host cell responses to pathogens. The bovine intestinal epithelial cell line was cultured in a 1:2 mixture of Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) (Seracare), 1M HEPES (Sigma H0887), gentamycin (Gibco 15750-060), apo-transferrin (Sigma T1428), epidermal growth factor (Sigma E9644), hydrocortisone (Sigma H0135) and insulin (Sigma I0516). Cells were seeded onto a 75x75 tissue culture flask (Corning CellBind #3290) and grown to confluence at 37°C under 5% CO₂.

5.2.2 Confirmation of Stx Receptor using Flow Cytometry

A more detailed protocol for receptor identification is outlined in Section 3.7. Briefly, after cell harvesting, cells were thoroughly resuspended in FACoLa and transferred to round bottom microtiter plates. Fifty microliters of the anti-CD77 monoclonal antibody was added to each well. FACoLa was added to each well and the plates were centrifuged. This was repeated two more times for a total of three washes. One hundred microliters of the goat anti-rat IgM fluorochrome conjugated secondary antibody (100µg, eBioscience #11-0990-82) was added to each well. A total of three washes were done again and at the end 2% formaldehyde in PBS was added to each well. Negative controls included cells with media only. The amount of specific antibody binding was calculated by subtracting the population of cells that bound an isotype-matched relevant monoclonal antibody (Table 3).

5.2.3 RNA purification

A total of 1.0×10^7 bovine intestinal epithelial cells were incubated in the presence or absence of Stx2 (2000CD₅₀, approximately 2000pg/ml) for 6 hours at 37°C under 5% CO₂. Cells were then harvested and washed twice with cold PBSA and resuspended in 1ml of Trizol (Sigma). Samples were stored at -20°C until RNA isolation. Upon thawing of samples, 200µl of chloroform was added and the samples were incubated at room temperature for 3 minutes, centrifuged for 10 minutes at 12000xg and the aqueous phase collected. Isopropanol was added

to each sample and incubated for 5 minutes at room temperature before applying to a Qiagen mini-column. RNA was isolated using the Qiagen RNeasy kit isolation protocol. Briefly, cells were centrifuged for 15 seconds at 9000 x g. DNase digestion was performed on column using a Qiagen DNase kit (Qiagen). To each sample, a total of 30U of DNase suspended in RNase- and DNase-free water was added to the column and incubated at room temperature for 15 minutes. The column was washed twice to remove the DNase using 350µl washing buffer (RW1). Another two washes to remove any remaining DNA were done using 500µl of buffer RPE. To elute RNA, 100µl RNase free water was added to the column and centrifuged at 9000 x g for one minute. The samples were stored immediately at -20 °C. The RNA concentration and integrity was determined by capillary electrophoresis on a Bioanalyzer (Agilent Technologies, Mississauga, ON).

5.2.4 cDNA Synthesis

The protocol for first-strand cDNA synthesis was followed according to the Manufacturer (Invitrogen). Briefly, 1µg of RNA was added to 10µl of 2X RT reaction mix and 2µl of RT enzyme mix. All incubations were done in a thermal cycler. The mixture was incubated at 25°C for 10 minutes followed by incubation at 50°C for 30 minutes. The reaction was then terminated at 85°C for 5 minutes. Nucleic acid content was estimated spectrophotometrically using the A_{260}/A_{280} ratio.

5.2.5 Quantitative RT-PCR (qRT-PCR)

Quantitative RT-PCR and data analyses were performed as described [205]. All qRT-PCR reactions contained 1µl target cDNA, 0.2µM each of forward and reverse primers (Table 6), and 9µl of qRT-PCR SuperMix (Invitrogen). Deionized, diethylpyrocarbonate-treated double distilled water was added to a final volume to 15µl. Reactions were run in duplicate in 96-well RT-plates covered with optical quality tape 170 (Bio-Rad) using the iCycler iQ Real-Time PCR detection system (Bio-Rad). Amplification was performed using the following protocol:

Cytokine Gene	Primer Sequence (5' → 3')
IFN γ	FWD-TCAAATTCGGTGGATGATCTGC REV-GACCATTACGTTGATGCTCTCCG
IL6	FWD-ATCAGAACACTGATCCAGATCC REV-CAAGGTTTCTCAGGATGAGG
IL12	FWD-ATGAGAGTTGCCTGGCTTCCAGAG REV-GCGTGAAGAAGGATGCAGAGCTTG
IL8	FWD-AGTGTGGCCCACTCTCAATAAC REV-CAAGAGCCAGAAGAAACCTGAC
TNF α	FWD-CTCTTCTGCCTGCTGCACTTC REV-CCATGAGGGCATTGGCATAACG
B-actin	FWD-AGGCATCCTGACCCTCAAGTA REV-GCTCGTTGTAGAAGGTGTGGT

Table 6. DNA primer sequences used for amplification of cytokine genes. The forward primer contained a ribosome binding site sequence and the starting sequence of each gene. The reverse primer contained the necessary sequence for amplification at low annealing temperatures. FWD: forward, REV: reverse

initial denaturation at 95 °C for 3 minutes (cycle one), followed by cycle two (95 °C, 15 seconds; 60 °C for 30 seconds; 76 °C for 30 seconds) which was repeated 45 times. Cycle 3 ran at 95 °C for 1 minute. Cycle 4 was preset at 45 °C ramping to 95 °C with 1 °C increase each 30seconds. The final hold temperature was 22 °C. Each plate had a negative template control that contained all the reagents except cDNA. The amplification of the product was determined by measuring the amount of SYBR Green I dye incorporated in the PCR product and plotted as fluorescence versus cycle number. The SYBR Green I dye is a double stranded DNA binding dye that emits fluorescence as the double stranded DNA is generated. The fluorescence intensity increases proportionally to the double stranded DNA concentration [206].

The relative difference in gene expression was calculated and represented as fold change $2^{-\Delta\Delta Ct}$ [206], where the Ct was defined as the cycle number at which the first detectable fluorescence increase above the threshold was observed. $\Delta Ct = Ct$ value of target gene minus Ct value of internal control (Ct value of β -actin rRNA). The $\Delta\Delta Ct = \Delta Ct$ of test minus ΔCt of control. The qRT-PCR reactions were carried out in duplicate and the data showed fold change in mRNA which is $2^{-\Delta\Delta Ct}$ [206].

5.2.6 Secreted Protein Preparation

A detailed protocol for isolating secreted proteins is described in section 3.4. Briefly, secreted proteins were isolated from a Stx2⁺ wild type *E. coli* O157:H7 (1961) and Stx2⁻ mutant *E. coli* O157:H7 strains (92192). Twenty millilitres of wild type Stx2⁺ *E. coli* O157:H7 and mutant Stx2⁻ *E. coli* O157:H7 were grown up to stationary phase overnight in LB (or LB + 15µg/ml gentamicin for mutant strain). Both the Stx2⁺ wild type *E. coli* O157:H7 strain and the Stx2⁻ mutant *E. coli* O157:H7 overnight cultures were subcultured the next morning and allowed to grow to log phase using constant aeration (OD₆₀₀ of approximately 0.7). The supernatants were concentrated as described [174].

5.2.7. Cytokine Analysis

A detailed ELISA protocol for determining cytokine production is described in section 3.6. Bovine intestinal epithelial cells were incubated with purified Stx2 (concentration of 2000CD₅₀) for 6 hours. After the incubation, the supernatant was removed from the tissue

culture flask. One hundred microliters of media was immediately applied to an IFN γ -specific pre-coated ELISA plate and diluted in a two-fold dilution series for cytokine concentration evaluation.

5.3 Results

5.3.1 Flow Cytometry of bovine intestinal epithelial cell line to confirm expression of Shiga toxin Receptor

To establish if the bovine intestinal epithelial cell line expresses Shiga toxin receptor (Gb₃), we used flow cytometry to measure the amount of anti-CD77/Gb₃ binding. Once the presence of surface Gb₃ was confirmed, we used the cell line to determine the effects of Stx2 on cytokine gene expression. The flow cytometry results are shown in Figure 12 and indicate that the bovine intestinal epithelial cells express the Stx receptor on their cell surface. We concluded that this is an appropriate cell line to use to investigate the effects of Stx2 on cytokine gene expression.

5.3.2 Cytokine profile of the bovine intestinal epithelial cell in the presence of purified Stx2.

To determine if Stx2 downregulates cytokine gene expression from intestinal epithelial cells, we tested the effect of purified Stx2 on the expression of selected cytokine genes by real-time PCR using a bovine intestinal epithelial cell line. The results of the qPCR analysis suggested lower cytokine gene expression of IFN γ , IL8, IL6 and IL12 in the presence of Stx2 (Figure 13).

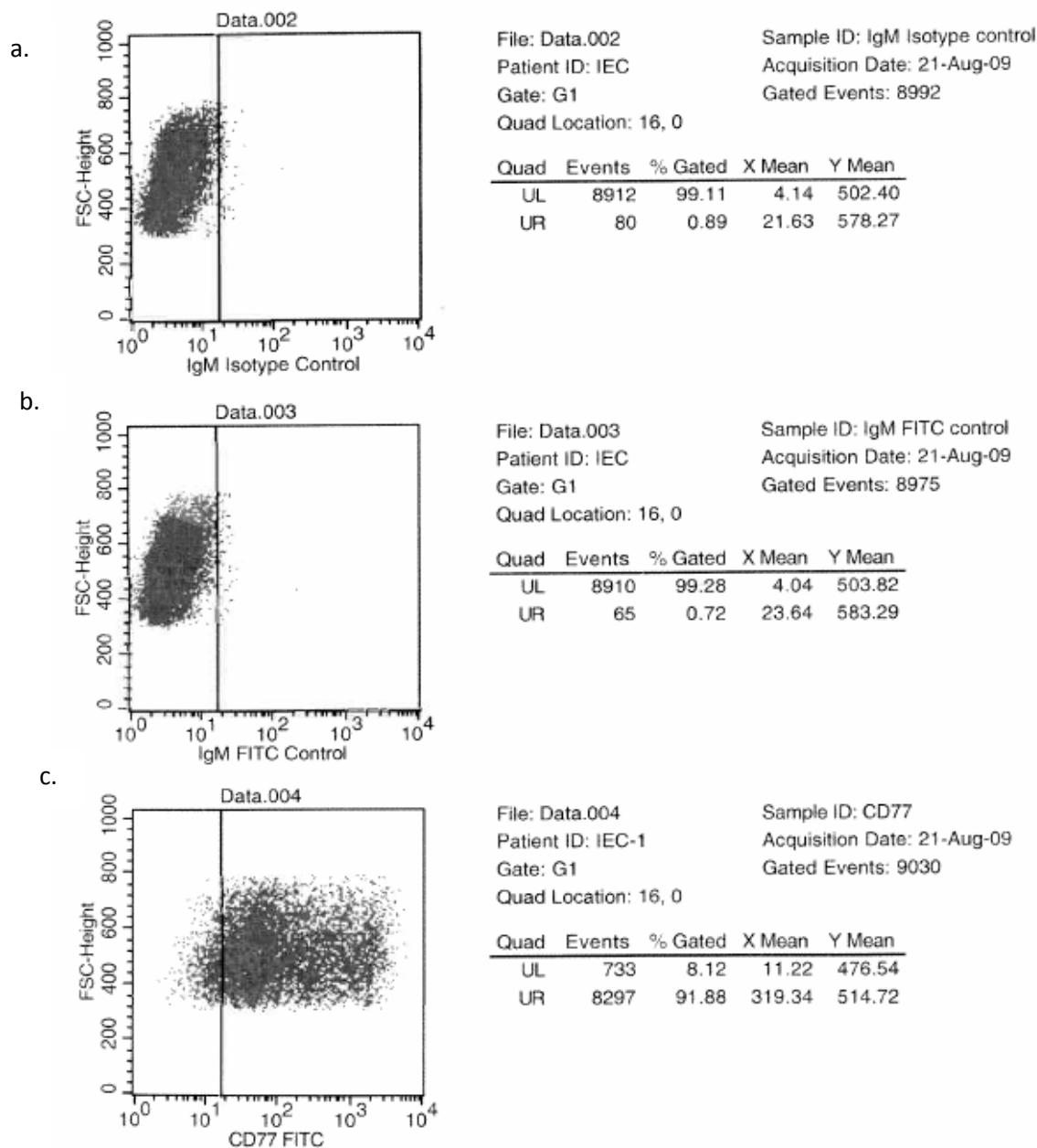


Figure 12. Flow cytometry analysis of the bovine intestinal epithelial cell line. An Anti-CD77 monoclonal antibody was used to identify c) CD77 receptor bearing epithelial cells. The appropriate controls (a) isotype and b) secondary antibody) were included on each 96 well plate and used to exclude any non-specific antibody binding that may occur during the analysis (Table 3).

Statistical analysis of cytokine gene expression in the presence of Stx2 showed it was not significantly different than cytokine gene expression in the absence of Stx2 for IFN γ , IL8, IL6, TNF α and IL12. The qPCR results are shown in Figure 13 and indicated that IFN γ cytokine gene expression was approximately 0.3 fold less than IFN γ gene expression of the bovine intestinal epithelial cell line incubated without toxin. IL6, IL12 and IL8 cytokine gene expression were approximately 0.8 0.7 and 0.5 fold less respectively when compared to gene expression in the absence of Stx2 (Figure 13). TNF α cytokine gene expression remained unaltered in the presence of purified Stx2. The β -actin gene was selected as an internal standard (loading control) as it is expressed in bovine cells. No significant difference ($P < 0.05$) in β -actin gene expression was found when cells were either in the absence or presence of Stx2. These observations suggest lower cytokine gene expression of IFN γ , IL6, IL12 and IL8 in the presence of Stx2.

5.3.3 Cytokine profile of bovine intestinal epithelial cells in the presence of Secreted protein preparations with and without Stx2.

STEC uses type III secreted proteins to colonize its hosts and Stx2 is a component of the secreted protein “cocktail”. Therefore, we wanted to determine if Stx2, in the presence of type III secreted proteins, modulates bovine immune responses through cytokine gene expression. To do this, secreted protein preparations were isolated from a wild type Stx2⁺ *E. coli* O157:H7 strain (1961) and a mutant Stx2⁻ *E. coli* O157:H7 strain (92192) and cytokine gene expression was analyzed by real-time PCR using a bovine intestinal epithelial cell line. The results of the qPCR analysis (Figure 14) indicated that cytokine gene expression of IFN γ , IL8, IL6 and IL12 was reduced in the presence of the secreted protein preparation isolated from the wild type Stx2⁺ *E. coli* O157:H7 strain when compared to cytokine gene expression of the bovine intestinal epithelial cell line incubated with the secreted protein preparation isolated from the mutant Stx2⁻ *E. coli* O157:H7 strain. Statistical analysis of the cytokine gene expression in the presence of the wild type Stx2⁺ *E. coli* O157:H7 strain was not significantly different than cytokine gene expression in the presence of the mutant Stx2⁻ *E. coli* strain for IFN γ , IL8, IL6, TNF α and IL12. The β -actin gene was selected as an internal standard (loading control) as it is expressed in bovine cells. No significant difference ($P < 0.05$) in β -actin gene expression was found when cells

were either in the absence or presence of Stx2. The data in Figure 14 suggested a trend to support the hypothesis that cytokine gene expression is downregulated in the presence of Stx2.

5.3.4 IFN γ production by bovine intestinal epithelial cells in the presence of purified Stx2.

To substantiate the results of the cytokine gene expression analysis, IFN γ -specific ELISAs were done to evaluate IFN γ protein concentrations resulting from incubation of the bovine intestinal epithelial cell line with purified Stx2. The results indicated that there was less IFN γ produced by the cells exposed to Stx2 compared to cells not exposed to Stx2 although statistical analysis using the Mann-Whitney test indicated that the levels of IFN γ were not significantly different (Figure 15). Approximately 800ng/ml of IFN γ was produced by the bovine intestinal epithelial cells not exposed to Stx2 and approximately 500ng/ml of IFN γ was produced by the cells exposed to Stx2. Why the cell line in the absence of any stimuli/mitogen produced the amounts of IFN γ observed has yet to be determined (Figure 15).

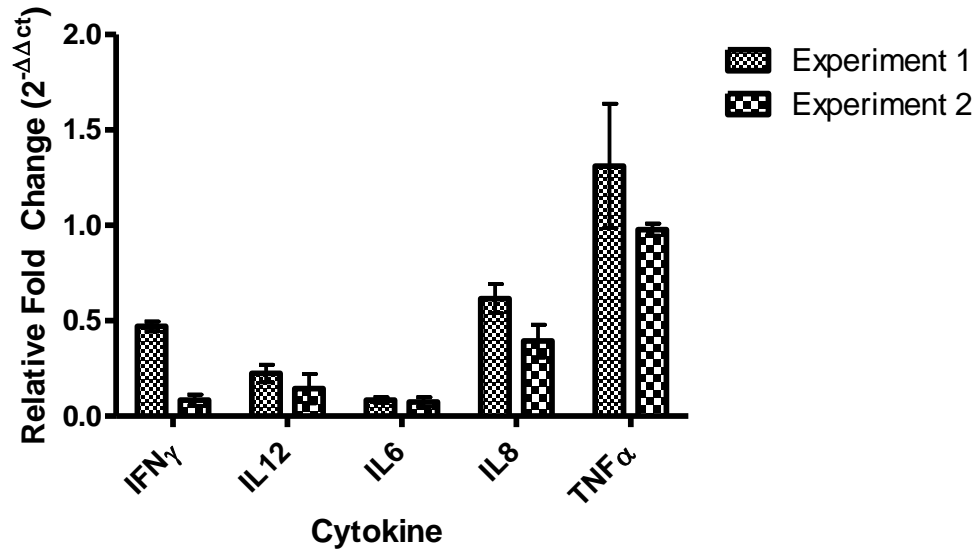


Figure 13. Cytokine gene expression levels in the presence of purified Shiga toxin 2. Bovine intestinal epithelial cells were incubated for 6 hours with purified Shiga toxin 2 (2000CD₅₀ as determined on VERO cells). After the 6 hour incubation, cells were collected, RNA extracted and cDNA was prepared. Each cytokine was normalized against β -actin gene expression in the presence and absence of Stx2. Data represents duplicate qPCR analysis of each cytokine gene from two independent experiments.

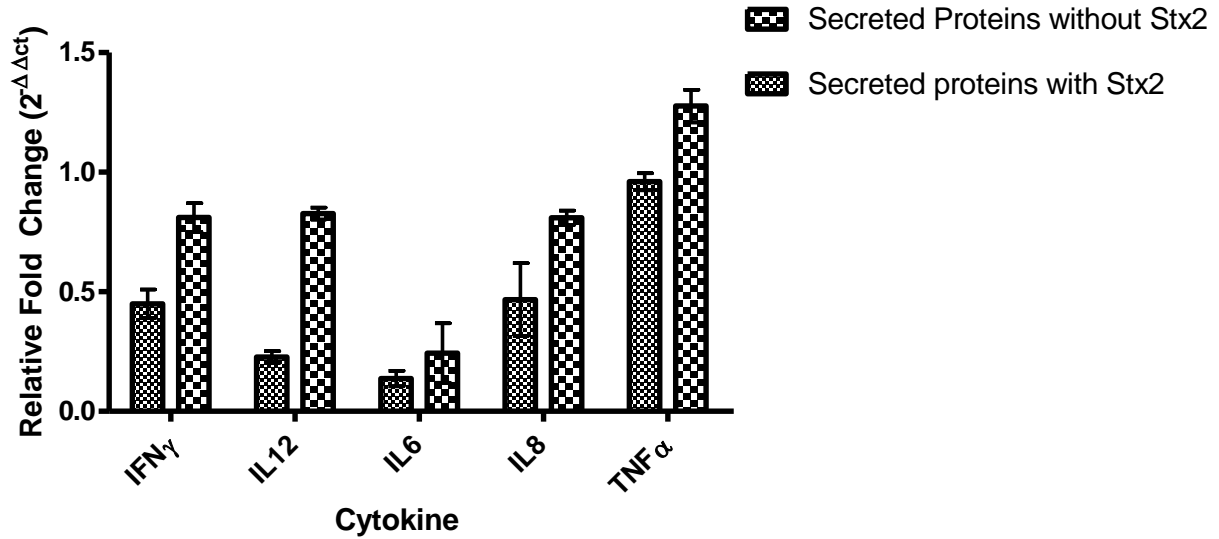


Figure 14. Cytokine gene expression in the presence of the secreted protein cocktails isolated from the wild type *Stx2*⁺ *E. coli* O157:H7 strain and the mutant *Stx2*⁻ *E. coli* O157:H7 strain. After 6 hours of incubation, bovine intestinal epithelial cells were collected, RNA extracted and cDNA was made. Each cytokine was normalized against β -actin gene expression in the presence and absence of *Stx2*. Data represents duplicate qPCR analysis of each cytokine gene from three independent experiments.

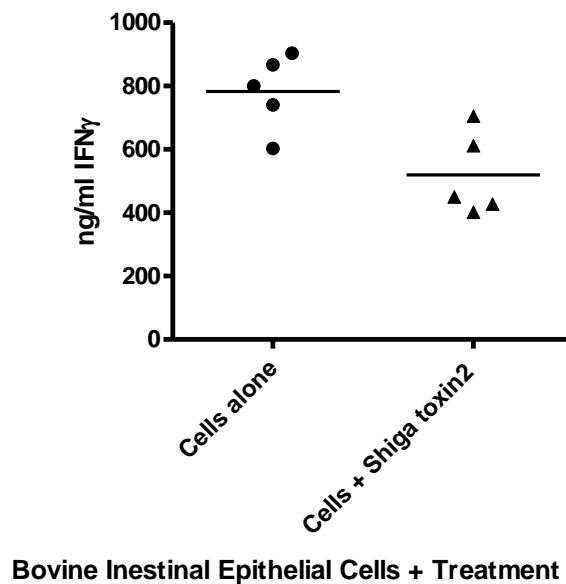


Figure 15. IFN γ production by bovine intestinal epithelial cells in the presence of Stx2.

The bovine intestinal epithelial cell line was split at a 1:2 ratio using the culture media described above and allowed to grow in a 75x75 tissue culture flask until confluent (96 hours at 37°C under 5% CO₂). On the fourth day, the purified Stx2 preparation was added to the tissue culture flask and allowed to incubate with the cells for 96 hours at 37°C under 5% CO₂.

5.4 Discussion

Mucosal surfaces are principal sites of entry for bacterial pathogens. The epithelial cells lining these mucosal surfaces are often the host's first line of defence providing a physical barrier between the pathogen and the host's internal environment [207]. In the gastrointestinal tract, the epithelial cells play an active role in producing signals that either induce or suppress the neighbouring immune and inflammatory cells in the mucosa [207]. Since STEC is a non-invasive pathogen, adherence to the intestinal epithelium is of particular importance for long term colonization within the bovine host. Published data regarding the expression of the Stx receptor on intestinal epithelial cells has been variable due to methodological constraints and exact lineage of cell lines. A number of reports have demonstrated the binding of Stx1 or the anti-CD77 antibody to the crypt regions of both bovine and human intestinal cell lines [85, 208, 209]. Hoey et al [208] observed Stx receptors on the surface of a bovine intestinal epithelial cell line and Stamm et al [178] described a bovine colonic cell line that is Stx receptor positive and bound rStxB1 molecules. In the present study, we also show Stx receptor expression on the surface of the bovine intestinal epithelial cell line using a monoclonal antibody to the CD77 antigen. The cell line used in the present study has been characterized and described by Kauschik et al [204] and has been confirmed suitable for studying host cell responses to pathogens.

Dean-Nystrom et al [210] showed that STEC strains are able to colonize the epithelium of the small intestine, the gall bladder, colon and the rectum of calves within 96 hours after oral inoculation. Therefore it has been hypothesized that STEC adherence to the intestinal epithelium is important for STEC colonization in cattle. *E. coli* O157:H7 has the ability to intimately attach to cells by rearranging the actin cytoskeleton using a type III secretion system found on the pathogenicity island called LEE. There is substantial amount of published data to support the hypothesis that *E. coli* O157:H7 uses LEE-encoded proteins to destroy tight junctions and intimately attach to intestinal cells [211]. But there is a growing amount of data revealing other factors independent of LEE that are required for colonization of the intestinal epithelium [212-214].

To date, there are numerous studies demonstrating that STEC modulates host immune responses in epithelial cells using a variety of mechanisms. STEC flagellin has been shown to be

a potent proinflammatory molecule *in vitro* but its expression *in vivo* has not yet been demonstrated. Yamasaki et al [215] showed Stx-dependent cytokine induction in a human colon epithelial cell line independent of bacterial LPS or any other bacterial component. As well, Thorpe et al [201, 202] have shown an induction of IL8 in the presence of Stx1 from a human colonic epithelial cell line. On the other hand, Hauf et al [216] have shown that EspB suppresses cytokine-induced NF- κ B activation. Similar experiments using HCT-8 cells did not demonstrate IL8 induction after 3 hours of incubation with purified Stx2 [202]. In the present study, we show a trend suggesting Stx2 suppresses cytokine induction at the transcriptional level in a bovine intestinal epithelial cell line.

The data suggests that induction of cytokines in humans may be of significance in the pathogenesis of HUS. Since cattle do not develop HUS or any clinical disease upon infection with STEC, it was our hypothesis that the role of Stxs in STEC-epithelial cell interaction results in the downregulation of bovine immune responses which is contrary to what is seen in humans. Therefore, the present study is an *in vitro* analysis of the effect of Stx2 on cytokine gene expression at the transcriptional level in bovine intestinal epithelial cells. The results in the present study indicate that induction of the cytokines IL12, IL6, IL8 and IFN γ from a bovine intestinal epithelial cell line was suppressed in the presence of Stx2. To date, there is limited published data to supplement our findings. Recently, Bellmeyer et al [217] demonstrated that a Stx⁺ EHEC strain suppressed inflammatory responses to cytokines and Stx itself compared to an Stx⁻ isogenic mutant strain. Also, Berin et al [218] failed to show an induction of IL8 in the presence of purified Stx2 from Caco-2 cells. Menge et al [219] showed that Stx1 perturbs the cytokine profile of bovine intraepithelial lymphocytes, immune cells situated within the gastrointestinal epithelium. Experiments using a bovine intestinal epithelial cell line showed Stx1 was unable to stimulate the release of granulocyte chemoattractants *in vitro* or induce intestinal inflammation *in vivo* [199, 200]. Phillips et al [167] have reported on STEC O157:H7 strains whose adhesion is restricted to the follicle-associated epithelium of ileal Peyer's patches. The implications of this finding could suggest that factors (such as the Stxs) expressed by STEC are conveniently situated near induction sites within the GALT in order to exert their immunomodulating effects [220]. Consequently these findings as well as the data presented here points to the possibility of an immunomodulatory role for Stxs within the bovine host to promote

STEC colonization and persistence. Despite the fact that there is very little published data regarding STEC-epithelial cell interactions, further understanding is crucial in order to develop effective strategies to reduce STEC shedding from the bovine host and thereby reduce human exposure and disease.

6.0 *In Vivo* Analysis of the Immunomodulatory Effects of Shiga toxin 2 using a Gut-loop Model

6.1 Introduction

Shiga-toxin producing *E. coli* (STEC) is a significant zoonotic pathogen causing a range of clinical symptoms that may lead to hemolytic uremic syndrome (HUS) if infection persists. The mortality rate for HUS is 5-10% [9, 81]. Hemolytic uremic syndrome is a consequence of the Shiga toxins (1 and 2) produced by STEC, which were initially described in *Shigella dysenteriae* [38].

The Shiga toxins are A-B proteins comprised of one A subunit to every five B subunits. The B subunits are responsible for recognizing and binding to the receptor, Gb₃, a glycosphingolipid having many different cellular roles. Apart from being a bacterial toxin receptor, Gb₃ is also a tumour and blood group antigen and a regulator of cell proliferation [58]. Traditionally the role of the Shiga toxins was thought to only be cytotoxic. The A subunit of the toxin is internalized via clathrin-coated pits where it is responsible for inhibiting protein synthesis. Recently, the role of the Shiga toxins has expanded and experiments suggest that they may also have an immunomodulatory role during STEC pathogenesis. In 2001, Menge et al [67, 68] showed that bovine lymphocytes isolated from the gastrointestinal tract and blood express functional Stx receptors on their surface. Lowered lymphocyte proliferative responses are seen *in vitro* in the presence of Stx1 with both human and bovine immune cells [68, 179]. However, the effect of Stx1 is believed to be restricted to B cells in humans but extends to both B and T cells in bovines [178, 179, 199].

To date, many *in vitro* studies have been done that indicate that bacterial products are involved in inhibiting the activity of host cells [221]. EspB, part of the type III secretion system of *E. coli* O157:H7, has been shown to suppress host NF- κ B activation and Menge et al have showed that Shiga toxins downregulate lymphocyte proliferation [68, 216]. Based on these results, it has been suggested that Shiga toxins are important virulence factors contributing to STEC persistence in cattle. Therefore, an *in vivo* investigation is crucial to our understanding of STEC immune suppression. We hypothesize that Shiga toxin 2 modulates the bovine mucosal immune system *in vivo*. To test this hypothesis, an *in vivo* model described by Gerdt et al [222]

using multiple intestinal loops surgically made in the jejunum. In young ruminants, the ileum is comprised almost exclusively of Peyer's patch tissue extending up to two meters in length [137]. Each loop included a portion of Peyer's patch tissue and served as an independent site to analyze the mucosal immune response to a wild strain of *E. coli* O157:H7 producing Stx2 and an insertionally inactivated Stx2⁻ *E. coli* O157:H7 mutant strain. In 2004 Menge et al [203] used intestinal loops to study the effects of Stx1 on ruminant intraepithelial lymphocyte populations and concluded that the ileal loop model is a useful tool to study mucosal responses to STEC in the bovine host. Results of the present investigation indicated that loops exposed to the wild type *E. coli* O157:H7 strain had a significant reduction in their CD4⁺ T cell population when compared to loops exposed to the Stx2⁻ *E. coli* O157:H7 mutant strain. Analysis of cytokine concentrations from the different loops failed to show a significant effect of the different *E. coli* O157:H7 strains. These results indicate that Stx2 may be an important immune modulator within the bovine host contributing to STEC pathogenesis.

6.2 Materials and Methods

6.2.1 Bacterial strains and growth conditions

The STEC strains used in this study were a wild type Stx2⁺ strain (1961) and an isogenic Stx2⁻ strain (92192) (obtained from Dr Carlton Gyles, University of Guelph, Guelph, Ontario, Canada). Previous characterization of the Stx2⁻ mutant strain 92192 revealed an insertion mutation in the *stx2* gene rendering the strain gentamicin resistant. The night before the surgery a single colony forming unit of the wild type *E. coli* O157:H7 strain was taken from an LB plate and inoculated into 20mls of Luria Broth (LB) (Gibco). Also, a single colony forming unit of the mutant *E. coli* O157:H7 grown up on an LB + 15µg/ml gentamycin plates was inoculated in 20mls LB + 15µg/ml gentamycin (Gibco #15750-060). The cultures were allowed to grow overnight to stationary phase at 37°C with constant aeration. The next morning, 3mls of each overnight culture was inoculated into 30mls of fresh LB +/- 15µg/ml gentamycin and the cultures were allowed to grow to log phase which is approximately an OD₆₀₀ of 0.7. At this time, 15mls of each log phase culture were removed and placed on ice for transport to the surgery room.

6.2.2 Animal Experiments

The animal experiment performed was in accordance with the University of Saskatchewan Committee on Animal Care which are consistent with the guidelines provided by the Canada Council for Animal Care. A 6 week old Holstein bull calf was fed twice daily with free access to water. The calf was observed daily prior to surgery and remained free of any clinical symptoms such as diarrhea. The calf was fasted for 12 hours prior to surgery.

6.2.3 Ligated Ileal Loop model

The bovine ligated jejunal loop model was described previously by Gerdtz et al [222] and a bovine ligated ileal loop model was previously employed by Menge et al [203]. This procedure was followed with minor modifications. Briefly, the calf was anaesthetized for the duration of the experiment (approximately 6 hours) by intravenous administration of pentobarbitone sodium. The calf was positioned in dorsal recumbence and a midline abdominal incision was made. Throughout the surgery, the ileum was constantly moistened with warm phosphate-buffered saline without calcium and magnesium (PBSA; pH7.2). A segment of the ileum was surgically prepared by removing ingesta and flushing twice with warm PBSA containing 250mg metronidazole. The antibiotic solution was removed with a PBSA flush. The ileal segment was further subdivided into loops which were approximately 6cm long. Each loop included Peyer's patch tissue which was grossly visible. Three loops were designated wild type loops and were inoculated with 5mls of 1×10^7 /ml wild type *E. coli* O157:H7 that produces Shiga toxin 2. Three loops were inoculated with 5mls of 1×10^7 /ml mutant *E. coli* O157:H7 that does not produce Stx2 and were designated mutant loops. Two loops were inoculated with saline and were designated control loops (total volume 5mls/per loop). The continuity of the ileum was re-established with an end-to-end anastomosis. The calf was euthanized with an overdose of anaesthetic 24 hours post surgery. The loops were collected and placed into individual glass beakers containing ice-cold CMF-HBSS/5% FBS/antibiotic/antimycotic solution. The interspace intestinal regions were removed from the intestinal loops and were not used in cell isolation.

6.2.4. Intestinal Peyer's Patch Lymphocyte Isolation

A detailed protocol for lymphocyte isolation is outlined in section 3.2.2. Briefly, immediately upon loop retrieval, intestinal Peyer's patch lymphocytes (IPPL) were isolated from all eight loops. The tissue was cut into pieces and washed for 15 minutes in CMF-HBSS/5% FBS/antibiotic/antimycotic/10mM DTT at 37°C. The tissue pieces were placed into a new flask containing CMF-HBSS/5% FBS /antibiotic/antimycotic/10mM EDTA for 60 minutes at 37°C. After 60 minutes, tissue pieces were washed for 180 minutes in CMF-HBSS /5% FBS/ 200U/ml collagenase 4/ 30µg/ml DNase I. The supernatant was centrifuged and the pellet was resuspended in PBSA. This was layered onto Ficoll-Hypaque and centrifuged. The buffy coat was collected and washed three times in PBSA + 5% FBS.

6.2.5 Flow Cytometry Analysis of Intestinal Peyer's Patches lymphocytes

After cell isolation, IPPL populations from each loop were resuspended in cold FaCola. One hundred microlitres of each cell preparation were transferred to round bottom microtiter plates at a final concentration of 1×10^6 cells/well. Fifty microliters of the monoclonal antibody specific for CD4 (VMRD CACT183A), CD8 (VMRD CACT80C), MHC class II (VMRD H34A), CDC11 (VMRD BAQ153A) and CD21 (AbD Serotec CC211/MCA1424G) was added to each well (Table 2). This was allowed to incubate on ice for 15 minutes. One hundred microliters of FaCola was added to each well and the plates were centrifuged for 2 minutes at 350xg. This was repeated two more times for a total of three washes. One hundred microliters of the fluorochrome conjugated secondary antibody (Becton Dickinson FITC-Goat anti-mouse IgG diluted to 1/50) was added to each well and incubated on ice for 15 minutes. A total of three washes were done again and at the end 200µl of 2% formaldehyde was added to each well to fix the samples. The plate was stored at 4°C until ready to be analyzed (no longer than 2 weeks). Ten thousand events were acquired for each sample. Negative controls included cells with media only. The amount of specific antibody binding was calculated by subtracting the population of cells that bound the isotype-matched relevant monoclonal antibody (Table 2).

6.2.6 Cytokine profile Analysis

A detailed protocol for cytokine quantitation is described in Section 3.6. The monoclonal antibodies to IFN γ , TNF α , IFN α and L12 were resuspended in coating buffer and applied to 96 round bottom plates. The appropriate biotinylated antibody was applied to each well. The reactions were stopped when the OD₄₀₅ of the first dilution of the standard curve reached an OD₄₀₅ of approximately 2.000.

6.2.7 PCR Analysis of Stx2⁺ Wild type *E. coli* O157:H7 strain and the Stx2⁻ Mutant *E. coli* O157:H7 strain

The extraction of genomic DNA from *E. coli* wild type Stx2⁺ strain (1961) and the isogenic Stx2⁻ strain (92192) were carried out as described [223]. The *stx2A* gene was amplified using primers based upon the *E. coli* O157H7 EDL933 sequence. The sequence of the forward primer (Stx2A_F) and reverse primer (Stx2A_R) were (5'-GTTGTTAGCTCAGCCGGACA-3') and (5'-TTTGCAGTAACGGTTGCAGA-3'), respectively. The *stx2A* gene was amplified by polymerase chain reaction (PCR). The PCR reaction was carried out as follows: denaturation at 96°C for 1 minute, then 25 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 60°C, extension for 1.5 minutes at 68°C and a final extension step for 5 minutes at 68°C.

6.3 Results

6.3.1 Confirmation of an insertional mutation within the *stx2A* gene in the Mutant *E. coli* O157:H7 strain.

Previous characterization of the Stx2⁻ mutant *E. coli* O157:H7 strain (92192) revealed an insertion mutation in the *stx2A* gene conferring gentamicin resistance. The Stx2⁺ wild type strain (1961) does not possess the transposon and is therefore gentamicin sensitive. The presence of the transposon in the Stx2⁻ strain was confirmed using PCR analysis. The *stx2A* gene is approximately 1000bps in size. The PCR primers were designed with 200bps of flanking DNA on each side of the gene (Figure 16). The *stx2A* gene from the mutant strain contains a gentamicin transposon that is approximately 540bps in size. Therefore the total PCR product is approximately 2000bps (Figure 16).

6.3.2 Immunophenotype of IPPL exposed to a Stx2⁺ Wild type *E. coli* O157:H7 strain and an Stx2⁻ Mutant *E. coli* O157:H7 strain

Eight ligated “loops” were created in one 6 week old Holstein bull spanning the entire length of the ileum. Each loop was inoculated with either the Stx2⁺ wild type *E. coli* O157:H7 strain, the Stx2⁻ mutant *E. coli* O157:H7 strain or sterile saline. Viable ileal IPPLs were isolated after 24 hours from each intestinal loop inoculated with the different *E. coli* O157:H7 strains. The immunophenotyping results indicated that the IPPL populations were similar in percentages when comparing the loops inoculated with either STEC strain or saline with the exception of one population of T cells. Statistical analysis, using the Dunn’s Multiple Comparison Test following one-way repeated ANOVA, indicated that the Stx2⁺ wild type *E. coli* O157:H7 strain significantly reduced the total number of CD4⁺ T cells ($P < 0.05$) compared to the loops inoculated with the Stx2⁻ mutant *E. coli* O157:H7 strain. Other lymphocyte populations were also assessed including CD8⁺ T cells, CD21⁺ B cells and dendritic cells (double stain for MHC II and CD11). No statistical differences could be detected in any of these lymphocyte populations when compared to loops exposed to the different STEC strains or saline (Figure 17).

6.3.3 Cytokine Production by IPPL populations exposed to an Stx2⁺ Wild type *E. coli* O157:H7 strain or an Stx2⁻ Mutant *E. coli* O157:H7 Strain

Cytokine concentrations in the ligated loops exposed to either the wild type or the mutant *E. coli* O157:H7 strains were assessed using capture ELISAs in order to determine if Stx2 modulates bovine immune responses through cytokine production. The results indicated no significant difference in production of TNF α , IFN α and IL12 when comparing loops inoculated with either the Stx2⁺ or Stx2⁻ STEC strains. We did observe elevated IFN γ , TNF α , IFN α and IL12 cytokine levels in the loops inoculated with either STEC strain compared to the loops exposed to sterile saline (Figure 19). Approximately 1800ng/ml of IL12 was detected in Stx2⁺ loops and approximately 1500ng/ml of IL12 was detected in Stx2⁻ loops. Both of these loops had an increase in IL12 levels compared to the loops exposed to sterile saline (approximately 600ng/ml) (Figure 19a). IFN α concentrations in the loops inoculated with either the Stx2⁺ or Stx2⁻ strains were 13pg/ml and 20pg/ml respectively (Figure 19b). Almost identical cytokine concentrations were observed for TNF α production when loops were exposed to either STEC

strain. The results indicate that TNF α and IFN α cytokine levels were elevated in the loops that were exposed to both types of *E. coli* O157:H7 strains compared to the loops that received only sterile saline (which were 1000pg/ml and 2pg/ml respectively). When analyzing the IFN γ cytokine concentrations, the results indicated that approximately double the amount of IFN γ was produced in loops exposed to the wild type Stx2⁺ *E. coli* O157:H7 strain when compared to loops exposed to the mutant Stx2⁻ *E. coli* O157:H7 strain (Figure 19d). Statistical analysis revealed that these values were not significantly different. The levels of IFN γ were much lower in loops exposed to saline compared to the loops inoculated with the Stx2⁺ and Stx2⁻ *E. coli* O157:H7 strains (Figure 19d).

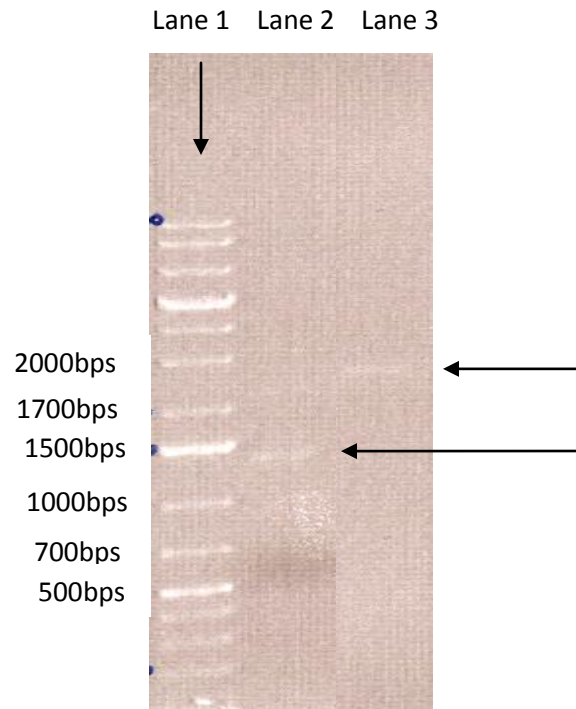


Figure 16. Polymerase Chain Reaction analysis of the A subunit of the *stx2* gene. Genomic DNA from the Stx2^+ wild type STEC strain and the Stx2^- mutant STEC strain was isolated and analyzed in order to confirm the presence of a gentamicin transposon in the *stx2A* gene of the mutant STEC strain. Arrows indicate lane 3 is the PCR product of the *stx2* gene isolated from the Stx2^- mutant STEC and lane 2 is the PCR product of the *stx2* gene isolated from the Stx2^+ wild type STEC.

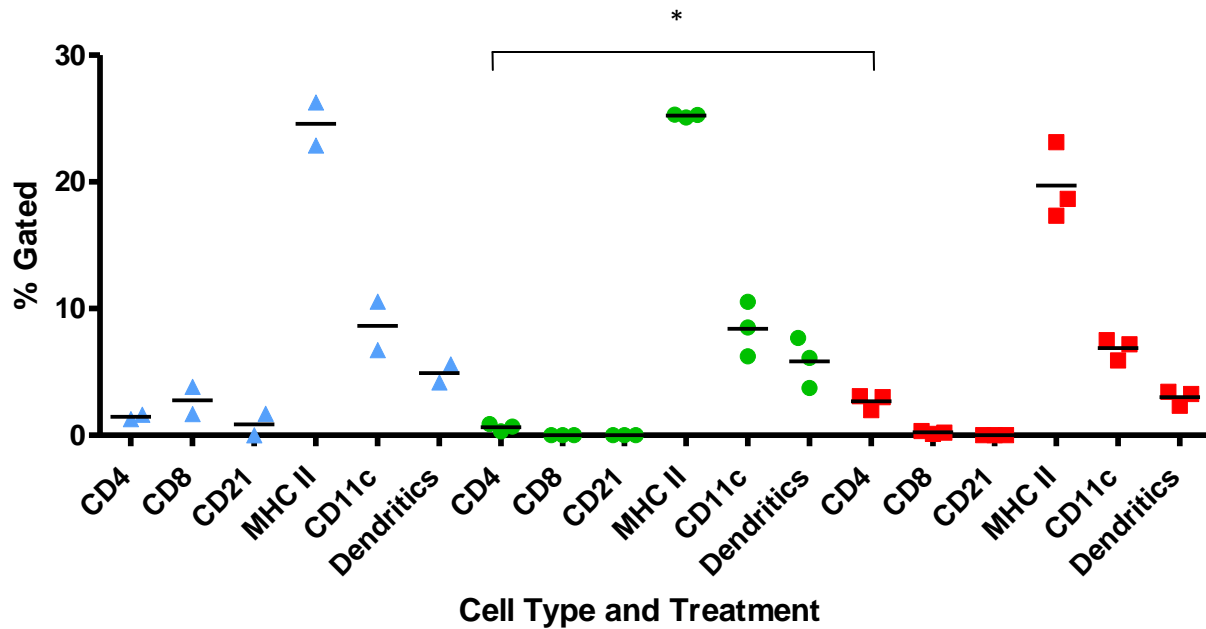


Figure 17. Immunophenotyping of the ileal IPPL populations isolated from the ligated loops. Freshly isolated IPPL populations from loops inoculated with a wild type Stx2^+ STEC strain (●), the mutant Stx2^- STEC strain (■), or saline (▲) were analyzed using flow cytometry. Statistical analysis using the Dunn's Multiple Comparison Test following one-way repeated ANOVA indicated that the number of CD4 T cells isolated from the loops exposed to the wild type Stx^+ STEC strain were significantly lower ($P < 0.05$) when compared to the number of T cells isolated from the loops exposed to the mutant Stx2^- STEC strain. * $P < 0.05$.

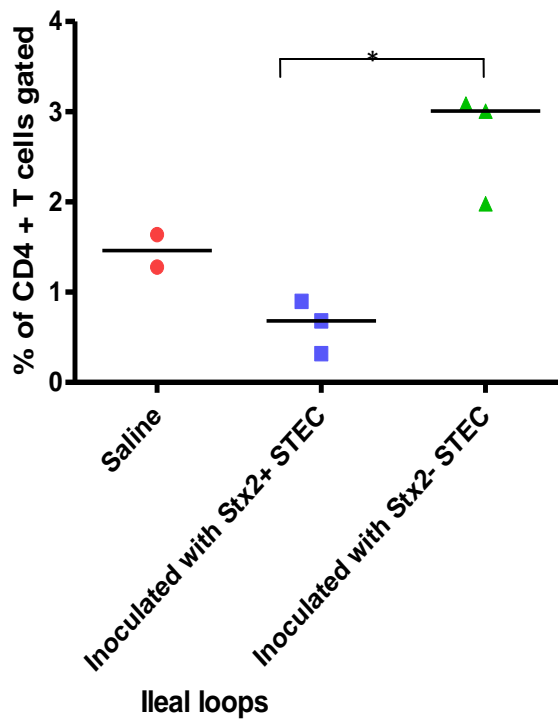


Figure 18. CD4⁺ T cell analysis of the ileal IPPL populations isolated from ligated loops. Freshly isolated IPPL populations isolated from loops inoculated with a wild type Stx2⁺ STEC strain (■), the mutant Stx2⁻ STEC strain (▲), or saline (●) were analyzed using flow cytometry. Ten thousand events were acquired for each sample. Statistical analysis using the Mann-Whitney test indicated that the number of CD4 T cells isolated from the loops exposed to the wild type Stx2⁺ STEC strain were significantly lower (P<0.05) when compared to the number of T cells isolated from the loops exposed to the mutant Stx2⁻ STEC strain.

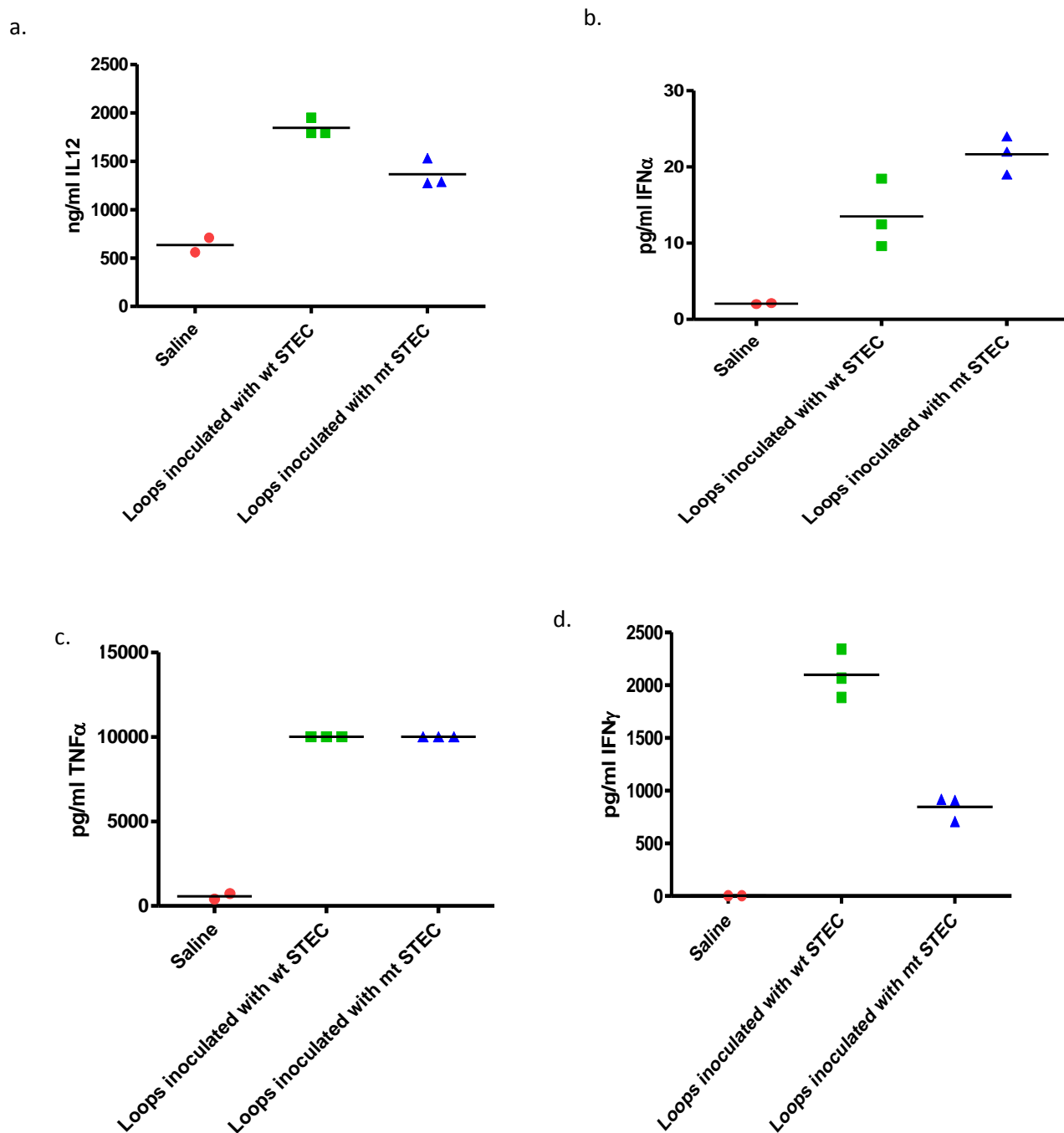


Figure 19. Cytokine production in ligated loops after exposure to Stx2. Cytokine production was measured after incubation with either wild type Stx2⁺ *E. coli* O157:H7 strain (■), the mutant Stx2⁻ *E. coli* O157:H7 (▲) or saline control (●). Supernatant collected from individual loops immediately after the calf was euthanized and applied to a) IL12, b) IL10, c) TNFα and d) IFNγ

specific ELISAs. The data points represent cytokine production from individual loops. Statistical analysis using the Mann-Whitney test to analyze loops inoculated with wildtype Stx2⁺ STEC and mutant Stx2⁻ STEC was done.

6.4 Discussion

It has been reported that calves inoculated with Stx2⁺ *E. coli* O157:H7 have significantly higher and longer fecal shedding of *E. coli* O157:H7 compared to calves inoculated with Stx2⁻ *E. coli* O157:H7 or a non-pathogenic *E. coli* [190]. Also, Stx2 can be detected in faecal samples of cattle and consequently probably achieves biologically active concentrations [224]. These results suggest that Stx2 may have a role in STEC pathogenesis within the bovine host. What remains to be explained is how Stx2 increases STEC pathogenesis thereby acting as a virulence factor during STEC colonization. The mechanism by which Stx2 facilitates STEC colonization is explained through the modulation of the bovine mucosal immune response. The depletion of bovine CD4⁺ T cells within the ileum implies that these lymphocytes are either direct or indirect targets of Stx2. In the present study, the CD4⁺ T lymphocytes were significantly reduced in the intestinal segments exposed to Stx2⁺ *E. coli* O157:H7 when compared to intestinal segments exposed to Stx2⁻ *E. coli* O157:H7. There was also fewer CD4⁺ T cells in the intestinal segments exposed to saline upon comparing the CD4⁺ T cell population exposed to Stx2⁺ *E. coli* O157:H7 which was not observed upon comparing with Stx2⁻ *E. coli* O157:H7 indicating an altered lymphocyte population in the presence of Stx2. This is in agreement with the role of Stx1 during STEC colonization. Menge et al [203] showed that Stx1 specifically targets bovine CD8⁺ intraepithelial lymphocytes within the ileum of calves. The results indicated a significant reduction in the percentage of CD8⁺ intraepithelial lymphocytes after 12 hours of incubation with a Stx1⁺ *E. coli* strain. *In vitro* studies have shown that Stx1 is also able to inhibit bovine peripheral lymphocyte proliferation suggesting a general immune suppression [68] although studies done by Johnson et al [102] have confirmed antibody responses to STEC antigens in cattle during experimental infections [102]. Therefore, a generalized immunosuppression in cattle by Shiga toxins has not been demonstrated. The present study provides direct evidence that Stx2 produced during STEC infection can modulate the specific lymphocyte population of the local mucosal immune system within cattle.

The gut-associated lymphoid tissue (GALT) is anatomically and functionally unique from the peripheral parts of the immune system. The GALT is constantly exposed to antigens in various different forms and has evolved the ability to distinguish between dangerous and useful antigens. Therefore it is of no surprise that the lymphocytes from the GALT also differ

phenotypically and functionally from peripheral lymphocytes. Lymphocytes activated in the GALT will recirculate back to mucosal tissue [136] and this is due to the fact that GALT-activated lymphocytes have a decreased expression of L-selectin and an increased expression of $\alpha_4\beta_7$ [136]. L-selectin is required for cell interaction with peripheral lymph nodes and $\alpha_4\beta_7$ interacts with MAdCAM-1 which is expressed on mucosal tissues [136]. STEC colonizes the gastro-intestinal mucosa while its products interact with the GALT. Dean-Nystrom et al [70] showed that calves inoculated with *E. coli* O157:H7 had A/E lesions in their rectum, colon and ileum suggesting STEC colonization in these three principle sites. To date, this is the first report on the effect of Stx2 on this unique population of lymphocytes *in vivo* within the bovine host.

Studies on Shiga toxins and cytokine induction/inhibition have proven difficult with results being variable due to species-specific and cell type-specific differences. In humans, data has suggested that Shiga toxins and cytokines work cooperatively to create the pathological changes observed during HUS [52]. Pro-inflammatory cytokines such as TNF, IL6 and IL8 have been shown to be elevated in the urine but not in the serum of HUS patients suggesting a renal source of production [198]. Louise and Keusch both showed that TNF α treatment of a human umbilical vein endothelial cell line and a human saphenous vein endothelial cell line increased sensitivity to Stxs possibly by increasing the number of Stx receptor *in vitro* [225, 226]. Van Setten et al [227] also showed that Stx1 increased synthesis of TNF α , IL6 and IL8 in nonstimulated freshly isolated human monocytes *in vitro*. Hughes et al [228, 229] showed an increase in proximal tubule TNF α production after exposure to Stx1 and also revealed that IL-1 increased Stx1 binding, but did not alter total cell amounts of Gb₃ or synthesis of Gb₃. In 1999 Yamasaki et al [215] showed a Stx-dependent cytokine induction in a human colon epithelial cell line independent of bacterial LPS or any other bacterial component. Therefore to date, the data suggests that induction of cytokines in humans may be of significance in the pathogenesis of HUS.

Cattle however do not develop HUS upon infection with *E. coli* O157:H7 but remain clinically normal [188]. Dean-Nystrom et al [70] showed in 1997 that *E. coli* O157:H7 strains were only pathogenic in neonatal calves approximately 12-36 hours of age and concluded that EHEC O157:H7 strains were not pathogenic in calves 3 weeks of age and older. Consequently, it remains to be determined if inflammatory processes play a significant role in cattle upon *E.*

coli O157:H7 infection. Menge et al [69] showed in 2003 that Stx1 does not alter IL2, TNF α or IFN α concentrations but rather modulates bovine immune responses by directly reducing lymphocyte populations. Moussay et al [199] also confirmed that Stx1 does not alter the amount of mRNA specific for IL2, IL10 and IFN γ in intraepithelial lymphocytes but did reveal a higher amount of IL4 specific mRNA. This effect was only specific for intraepithelial lymphocytes as the same experiment with PBMCs did not produce the same result [199]. In the present study, cytokine production was determined in the gut after 24 hours of exposure to wild type Stx2⁺ *E. coli* O157:H7 and mutant Stx2⁻ *E. coli* O157:H7. The results indicated that TNF α and IFN α cytokine levels were elevated in the loops that were exposed to both types of *E. coli* O157:H7 strains compared to the loops that received only sterile saline. The TNF α and IFN α cytokine concentrations though did not differ significantly when comparing the loops inoculated with either Stx2⁺ or Stx2⁻ strains. A similar trend was also observed with IL12 concentrations. When comparing the ligated loops inoculated with either the Stx2⁺ or the Stx2⁻ *E. coli* strain, there was no significant difference between the amounts of IL12 produced but both of these loops had an increase in IL12 levels compared to the loops exposed to sterile saline. There was a significant increase in IFN γ levels in the loops inoculated with the wild type Stx2⁺ *E. coli* O157:H7 strain when compared to the loops inoculated with the mutant Stx2⁻ *E. coli* O157:H7 strain. When comparing the loops exposed to sterile saline, the levels of IFN γ were lower compared to the loops inoculated with the Stx2⁺ and Stx2⁻ *E. coli* O157:H7 strains. Therefore these results indicate that the presence of STEC does trigger the bovine immune response to produce cytokines as all of the loops inoculated with either *E. coli* strain did produce elevated cytokines compared to loops exposed to sterile saline. Since none of the cytokine concentrations differed significantly between the loops inoculated with either *E. coli* strains though, we cannot attribute the increase in cytokine concentrations to Stx2 but rather to the bacteria itself and/or bacterial products such as LPS. Consequently, the results indicate that Stx2 does not modulate the bovine immunological response through a cytokine-mediated effect but rather on a specific lymphocyte population situated in the gastro intestinal tract. The present study confirms the growing amount of published data that demonstrates bovine immune cells are a predominant target for Stx2.

7.0 Immunomodulatory Effects of Shiga toxin 2 during Active Immunization

7.1 Introduction

Shiga toxin-producing *E. coli* (STEC) is an important food- and water-borne pathogen of humans, causing a variety of symptoms of which Haemolytic Uremic Syndrome (HUS) is the worst. The mortality rate for HUS is 5-10% [9, 81]. The link between HUS and STEC infection is well understood. In order to reduce the incidence of human exposure and thus disease, it is imperative to reduce the amount of STEC that is shed from principle animal reservoirs. Cattle are the primary non-human reservoir for STEC and are able to shed STEC for long periods of time without showing any clinical disease.

A number of prevention and treatment strategies are being studied in order to reduce the amount of STEC in cattle thereby reducing exposure and risk to humans. Antibiotic treatment in cattle is controversial due to the problem with bacterial resistance. Therefore much effort has been put into reducing the survival and transmission of STEC by focusing on farm management, disinfectant practices, probiotics and bacteriophage use within cattle [162]. On-farm interventions have been exploited including chlorination of water, general disinfection of living quarters, switching grain-fed cattle to an all hay diet prior to slaughter and quarantining animals until they test negative [157]. General disinfection and chlorination of water produced less than desirable results and quarantining animals proved impractical due to the transient nature of faecal shedding [155]. Finally, vaccination of cattle to reduce STEC shedding is being investigated by numerous scientific laboratories.

Since cattle are associated with human STEC infections, they serve as a logical vaccine target as a means to reduce human infections [162]. Several vaccine trials have been attempted with very promising results. In 2004 Potter et al [162] successfully reduced colonization of STEC by vaccinating with type III secreted proteins. Vaccination results showed a significant reduction in the number of bacteria shed in feces, the numbers of animals that shed, and the duration of shedding in an experimental challenge model. In 2005, Peterson et al [230] performed a single commercial feedlot trial also to evaluate the effects of vaccinating cattle with type III secreted proteins. Results of the trial showed that vaccination reduced the proportion of cattle shedding *E. coli* O157:H7 in their feces. One of the most recent vaccine trials published in

2009 by Smith et al [169] used 3683 cattle and a type III secreted protein vaccine. In order to detect shedding *E. coli* O157:H7, the terminal rectal mucosa was swabbed 3-5cm proximal to the rectoanal juncture. The results showed only 11 of 382 vaccinated animals were positive for *E. coli* O157:H7 revealing that vaccinated cattle were significantly less likely to be colonized with *E. coli* O157:H7 compared to nonvaccinated cattle [169]. Even though published data exists supporting vaccination in cattle as a means to reduce STEC, commercially available vaccines are not being used by farms and STEC continues to persistently colonize cattle and remain a significant zoonotic pathogen of humans causing disease and death.

The secreted proteins used in the above vaccine trials have all been isolated from Stx2⁺ *E. coli* O157:H7 strains [162]. Stx2 is an A-B protein comprised of one A subunit for every five B subunits. The B subunits are responsible for recognizing and binding to the receptor, Gb₃. Gb₃ is a glycosphingolipid ubiquitously found on eukaryotic membranes. Traditionally the role of the Shiga toxins was thought to only be cytotoxic. The A subunit of the toxin is internalized via clathrin-coated pits where it is responsible for inhibiting protein synthesis. Epidemiological and experimental data have revealed that Stx2 is more relevant than Stx1 [231]. For example, Stx2 is about 1,000 times more toxic for human renal microvascular endothelial cells than Stx1 [49]. The Stx2 family consists of Stx2, Stx2c, Stx2c2, Stx2d, Stx2e, and Stx2f [50]. Immunization trials using rabbits and pigs have shown that the Stx2 toxoid is protective upon further Stx2 holotoxin challenge [232, 233]. Studies using the B subunit of Stx2 as a vaccine candidate have been ongoing [56] but problems with large-scale production have arisen due to the instability of the B subunits without the A subunit. Although one study by Marcato et al [56] showed that rabbits immunized with the B subunit survived a subsequent challenge with the Stx2 holotoxin.

Reduced numbers of intraepithelial lymphocytes after exposure to Stx1 *in vivo* have been reported by Menge et al [203]. Similarly, Smith et al [234] have reported a depletion of lymphocyte numbers in the submucosal lymphoid follicles in five week old calves infected with an STEC strain. *In vitro* experiments have also demonstrated reduced lymphocyte proliferation in the presence of purified Stx1 [68]. These results have led to the hypothesis that the Shiga toxins may have immunomodulating effects in cattle and as a consequence, immunity to STEC as well as other co-administered vaccines may not be optional. Therefore we wanted to determine if Stx2 modulates bovine immune responses during active immunization in cattle. We

hypothesize that bovine immune response to a heterologous antigen such as hen egg lysozyme (HEL), will be downregulated during active immunization in the presence of Stx2. Two trials will be presented with the first trial using a purified Stx2 preparation. Bovine humoral and cellular immune responses to the academic antigen, HEL were evaluated. The second trial used secreted protein preparations isolated from a wild type Stx2⁺ *E. coli* strain and a secreted protein preparation isolated from a mutant Stx2⁻ *E. coli* strain. Again bovine humoral and cellular immune responses to the commercial antigen, HEL were evaluated. The results from both trials indicate that Stx2 downregulates peripheral bovine immune responses during active immunization.

7.2 Materials and Methods

7.2.1 Trial Design

To determine the effect of Stx2 on induction of bovine immune responses to heterologous antigens, immune responses to hen egg lysozyme (HEL) (Sigma #L6876) were determined using adult Holstein cows immunized with 500µg HEL with or without Stx2. HEL was formulated with 30% VSA3 as the adjuvant and a total of 2mls was administered subcutaneously in the neck of each animal. In each trial, vaccinations occurred on days 0 and 21. Serum samples were collected on days 0, 6, 10, 14, 21, 28 (35 if relevant). Bovine proliferative responses were analyzed on days 14, 21, 28 (and 35 if relevant) using freshly isolated PBMCs.

7.2.1.1 Trial 1: Antigenic Dosing of HEL

To establish the effect of Stx2 on induction of bovine immune responses to HEL, an appropriate antigenic dose of HEL was determined first in the absence of Stx2 using a 35 day trial consisting of four adult Holstein cows divided into two groups. Group 1 received 250µg HEL + 30% VSA [235] and group 2 received 500µg HEL + 30% VSA [235]. Samples were taken from each cow on the days noted above.

7.2.1.2 Trial 2: Immune responses to HEL immunization in the presence and absence of Stx2

Immune responses to hen egg lysozyme (HEL) were compared in the presence and absence of Stx2 to establish the effect of Stx2 on bovine immune responses. This was determined by immunization with either HEL alone or HEL coadministered with Shiga toxin 2. Therefore group 1 received 500µg HEL, 30% VSA [235] and 2µg Shiga toxin 2 per dose and group 2 received 500µg HEL and 30% VSA per dose [235]. The trial consisted of two groups of 8 adult Holstein cows and the duration of the trial was 30 days. The blood samples were taken from each cow on the days noted above.

7.2.1.3 Trial 3: Secreted protein trial

Immune responses to HEL and secreted proteins were determined in the presence and absence of Stx2 to determine the effect of Stx2 on the induction of bovine immune responses. Three groups of five cows were chosen and the vaccine formulations (per cow per dose) included group 1 (wild type group) which received 500µg HEL + 50µg secreted protein cocktail from wild type Stx2⁺ *E. coli* O157:H7 + 30% VSA [235], group 2 (mutant group) which received 500µg HEL + 50µg secreted protein cocktail from mutant Stx2⁻ deficient *E. coli* strain + 30% VSA [235] and group 3 (mutant + Stx2 group) which received 500µg HEL + 50µg secreted protein cocktail from mutant Stx2⁻ deficient *E. coli* + 30% VSA [235] + 2µg purified Stx2 (Section 3.1). The trial consisted of three groups of 5 adult Holstein cows and the duration of the trial was 35 days. The blood samples were taken from each cow on the days noted above.

7.2.2 Secreted Protein Preparations

A detailed protocol for isolating secreted proteins is described in section 3.4. Briefly, secreted proteins were isolated from the Stx2⁺ wild type *E. coli* O157:H7 strain (1961) and the Stx2⁻ mutant *E. coli* O157:H7 strain (92192). Twenty millilitres of wild type Stx2⁺ *E. coli* O157:H7 and mutant Stx2⁻ *E. coli* O157:H7 strains were grown up to stationary phase overnight in LB (or LB + 15µg/ml gentamicin for mutant strain). Both the Stx2⁺ wild type *E. coli* O157:H7 strain (1961) and the Stx2⁻ mutant *E. coli* O157:H7 (92192) overnight cultures were subcultured the next morning by inoculating 5mls into 500mls of minimal medium (M9 solution

(Sigma-Aldrich, St. Louis, MO, USA) [175] supplemented with 0.5M MgSO₄ + 5% casamino acids + 0.5% glucose + 1% NaHCO₂) and allowed to grow to log phase using constant aeration (OD₆₀₀ of approximately 0.7). Cultures were centrifuged twice removing the supernatant each time. The supernatants (containing the secreted proteins) were concentrated using a 10K Amicon filter (Fischer) and nitrogen gas at 4°C as described [174].

7.2.3 Western Blot Analysis of Stx2

The secreted proteins were isolated from the Stx2⁺ wild type *E. coli* O157:H7 strain (1961) and the Stx2⁻ mutant *E. coli* O157:H7 strain (92192). The proteins were separated by PAGE and transferred to a nitrocellulose membrane by electroblotting as recommended by the manufacturer (BioRad Laboratories) with a few modifications. The resolving gel and stacking gel were made as usual however SDS was omitted from the mixture. The 2X buffer also did not contain SDS or DTT. The secreted proteins were not boiled prior to electrophoresis as recommended by the Stx Western Blot SOP. Western blot analysis was then carried out using mouse monoclonal anti-Stx2 (OEM Concepts MAV67-212, 1mg/ml diluted to 1µg/ml for Western analysis). After incubation (for approximately 3 hours) with primary antibody, the membrane was washed three times with PBS-T and incubated with a secondary antibody conjugated to alkaline phosphatase for approximately 1 hour (Goat anti-mouse IgG (H+L) (KPL, Gaithersburg, MA, USA).

7.2.4 Enzyme-linked Immuno-Sorbent Assay (ELISA)

Hen egg lysozyme was prepared in coating buffer at 100µg/ml and 100µl of this solution was used to coat each well of a 96-well plate. The plates were covered with a lid, wrapped with saran wrap and placed inside a plastic bag to avoid drying, and incubated overnight for 16 hours at 4 °C. After incubation, the coating solution was removed and plates were washed 5 times with phosphate-buffered saline (PBS, 0.85% NaCl (w/v), pH7.0) containing 0.5% (v/v) of Tween 20 (PBST). The wells were blocked with 200µl/well of PBS-0.5% Tween 20 containing 0.03% gelatine (v/v) (PBST-g) for 2 hours at room temperature. Both serum and positive controls were serially diluted in four-fold increments in PBST-g and incubated for 1 hour. After five washes, alkaline phosphatase-conjugated polyclonal goat anti-bovine IgG and monoclonal sheep anti-

bovine IgG1 and IgG2 (Kirkegaard and Perry laboratories, Gaithersburg, MD, USA) were diluted to 1:5000 in PBST-g and 100µl of diluted antibody was added to each well and incubated for 1 hour. After five washes, 100µl of freshly prepared para-nitrophenyl phosphate (PNPP, 1mg/ml, Sigma #N3254) substrate solution in DE buffer (10.5ml of 1 M Diethanolamine, 1ml of 0.5 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 990ml of distilled water) was added per well and incubated for 45 minutes at room temperature. Finally using a microtitre plate reader, the absorbance was read at 405nm with reference set at 490nm using the SOFTmax Pro program.

7.2.5 IFN γ ELISPOT assay

The IFN γ ELISPOT protocol was carried out over three days. On day one, the anti- IFN γ antibody was diluted 1/3000 in sterile coating buffer and millipore HA plates were coated with 100µl per well. The millipore HA plates were wrapped in Saran wrap and refrigerated overnight at 4°C.

The morning of the second day, the millipore HA plates were washed in sterile PBSA four times. The millipore HA plates were blocked using 100µl of 1% albumin/PBSA and were incubated for 1 hour at 37°C. The blocking media was removed after 1 hour and 100µl of antigen (HEL, concentration 100µg/ml) was added to each well resulting in a final HEL concentration of 10µg/ml per well. At the same time, 100µl of freshly isolated bovine PBMCs were added to each well at a concentration of 10^7 /ml and the plates were incubated overnight at 37°C. Negative (media) controls and positive Concavallin A (ConA) controls were used on each Millipore HA plate.

On the third day, the millipore HA plates were submerged in double distilled H₂O (ddH₂O) for 5 minutes to lyse all cells and then washed three times in PBST and twice in double distilled H₂O. Rabbit anti-bovine IFN γ was diluted 1/1500 in 1% albumin/PBSA and 100µl added to each well and allowed to incubate at room temperature for 2 hours. The millipore HA plates were washed five times in PBST and twice in double distilled H₂O. After washing was completed, 100ul of substrate nitroblue tetrazolium (NBT) salt and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) was added to each well. The millipore HA plates were allowed to develop for a maximum 30 minutes then were washed in ddH₂O and allowed to air dry for 24 hours. The spots were counted manually using a dissecting microscope.

7.2.6. Proliferation assay

Lymphocyte proliferation was determined by adding 3×10^5 cells/well of freshly isolated bovine PBMCs (Section 3.2.1) into a round bottom 96 well plate and incubating for 96 hours at 37°C under 5% CO₂ with 10µg/ml HEL. On the fourth day, 20µl of 0.4µCi of [³H]-thymidine was added to each well. The plate was allowed to incubate for 6 hours at 37°C under 5% CO₂. Following this, the plates were immediately frozen until the incorporation of [³H]-thymidine could be assessed using a scintillation counter.

7.2.7 Cytokine ELISAs

A detailed protocol for cytokine quantitation is described in Section 3.6. Briefly, a monoclonal anti-IL4 antibody (Table 2) was resuspended in coating buffer and 100µl applied to a 96 round bottom plates and allowed to incubate at 4°C overnight, then the plates were washed four times with TBST and 100µl of each sample was applied to the plates and diluted two-fold. The plates were washed four times with TBST and the appropriate biotinylated antibody (Table 2) was applied to each well. The plates were allowed to incubate for one hour at room temperature followed by four washes with TBST. One hundred microliters of streptavidin alkaline phosphatase (diluted 1/5000) was added to each well for one hour. The plates were washed again in TBST and 100µl of PNPP substrate was added to each well. The reaction was stopped with 0.3M EDTA when the OD₄₅₀ of the first dilution of the standard curve reached an OD₄₅₀ of approximately 2.000.

7.2.8 Western Blot Analysis of Secreted Protein Preparations

A detailed protocol for western blotting is described in Section 3.4. SDS polyacrylamide gel electrophoresis of secreted proteins was carried out to confirm the presence and absence of Stx2 in the secreted protein preparations isolated from the wild type Stx2⁺ *E. coli* O157:H7 strain and the mutant Stx2⁻ mutant *E. coli* O157:H7 strains. Briefly, total protein was loaded onto an SDS-PAGE gel. The proteins were separated at 120 volts in a gel apparatus (Bio-Rad) for approximately 1 hour. For immunoblotting, proteins were transferred from the gel to a nitrocellulose membrane (Bio-Rad) using a power supply (PowerPac200, Bio-Rad). After the proteins were transferred to the nitrocellulose, the membrane was removed from the apparatus

and blocked with 1% milk overnight at 4 °C. After overnight incubation, the membrane was washed three times with PBS-T and incubated with a cocktail of primary antibodies raised against secreted proteins in a rabbit (produced at VIDO by D. Asper). After incubation with the primary antibodies, the membrane was washed three times with PBS-T and incubated with a secondary antibody conjugated to alkaline phosphatase (Goat anti-rabbit IgG (H+L) (KPL, Gaithersburg, MA, USA). After incubation with the secondary antibody, the membrane was washed once with PBS-T and additional once with alkaline phosphatase (AP) buffer. Finally, the membrane was incubated with the substrate, NBT/BCIP until color developed.

7.2.9 Cytotoxicity Assay

The cytotoxic activity of the secreted protein preparations were determined as described by Gentry et al [182]. Briefly, 50µl of a10-fold dilution series of the secreted protein preparations were applied to a 96 well plate containing 0.15M NaCl. Fifty microliters of cell culture media containing 10% FBS, 2mM glutamine, 100U penicillin and 100mg/ml streptomycin was added to each well and the plates were incubated for 30 minutes at room temperature. Following this, 50µl of VERO cells were added to each well at a concentration of 8×10^5 cells/ml and incubated at 37°C under 5% CO₂ for 96 hours. Cellular metabolic activity was measured using the MTT Reduction Assay as described [183].

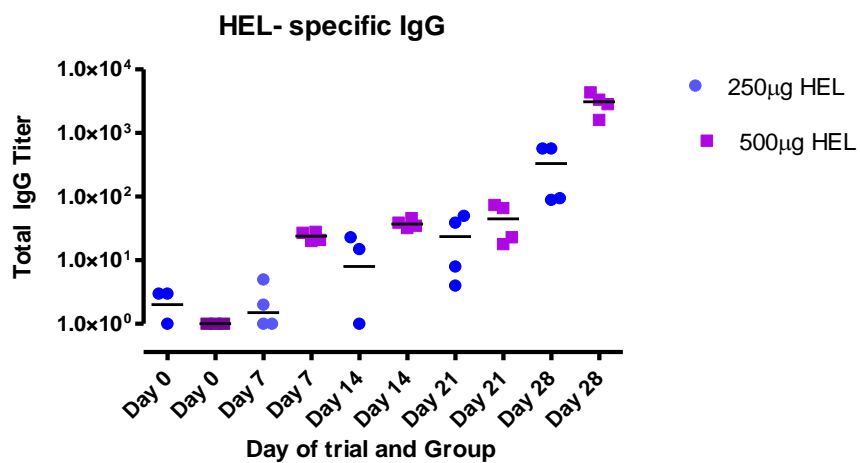
7.3. Results

7.3.1. Humoral and Cellular Immune responses to Hen Egg Lysozyme (HEL)

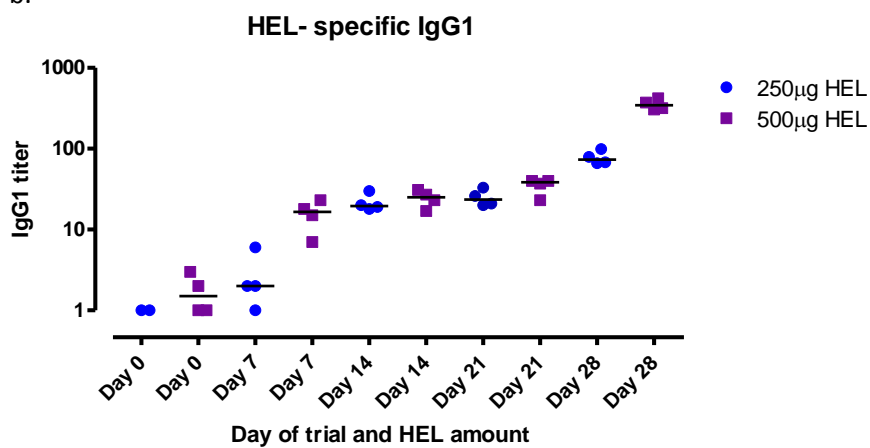
To measure bovine immune responses to the commercial antigen, HEL, an appropriate antigen dose was required to induce an immunological response large enough to be measured but not too strong to mask any potential effect of Stx2. Therefore, a pilot trial was done using four Holstein cows which were divided into two groups (outlined in section 7.2.1.1). The cows were given either 250µg HEL or 500µg HEL 21 days apart and serum and PBMCs were sampled to determine antibody and proliferative responses. The results of the trial indicated that the cows receiving 500µg of HEL produced antibody responses (total IgG, IgG1 and IgG2) that were numerically higher than the cows that received 250µg of HEL. Very little HEL-specific antibody could be detected on day 7 of the trial in the cows that received 250µg HEL but the cows that received 500µg of HEL had a 10-fold higher response for all three antibody isotypes tested. On days 14 and 21, total IgG, IgG1 and IgG2 antibody titers of both groups were almost identical. On the last day of the trial, the cows that received 250µg HEL had a median IgG antibody titer slightly above 100 and the cows that received 500µg HEL had a median antibody titer of slightly over 1000 (Figure 20a). Therefore these results suggested that 500µg HEL was a more appropriate antigenic dose for HEL because the total IgG response was greater in this group but not overwhelming so that it might mask any effect of Stx2 in the later trials.

To measure bovine proliferative responses to HEL, two concentrations of HEL (1µg/ml and 10µg/ml) were used to determine the appropriate *in vitro* restimulation concentration (Figure 21). The results indicated that cells restimulated with 10µg/ml compared to cells restimulated with 1µg/ml showed proliferative responses that could easily be measured using the incorporation of [³H]-thymidine suggesting that this was an effective HEL concentration. The results also indicated that cells from cows receiving 500µg HEL produced a proliferative response that were similar to the cows which received 250µg HEL when 10µg/ml HEL was used as the restimulation concentration. The results indicated that cells from cows which received 250µg HEL and 500µg HEL gave variable results when restimulated with 1µg/ml. We concluded based on both the humoral and cellular responses, that future trials determining the effects of Stx2 during active immunization would use 500µg HEL as the antigenic dose and 10µg/ml HEL for restimulation assay.

a.



b.



c.

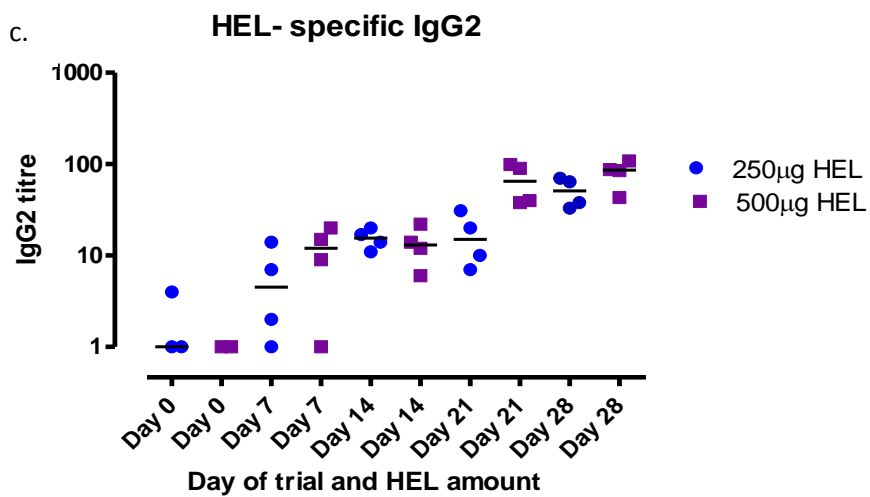


Figure 20. Serological responses following vaccination with HEL. The a) total IgG antibody activity to HEL, b) IgG1 antibody activity to HEL and c) IgG2 antibody activity to HEL during the course of a 28 day trial in which cows received either 250µg HEL (●) or 500µg HEL (■) were measured using ELISA as described above. The trial consisted of four animals, two animals per group and each data point represents a duplicate antibody titre.

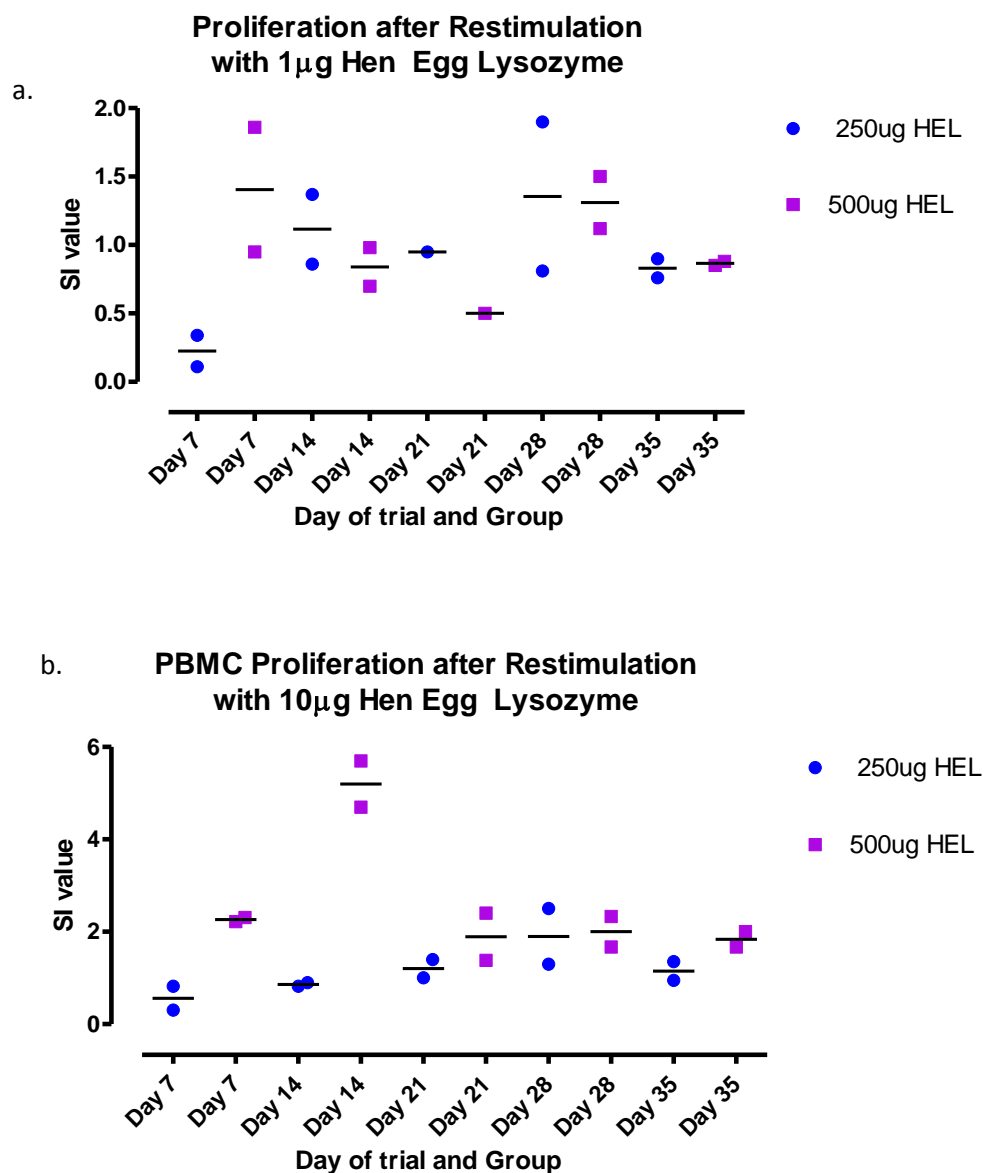


Figure 21. Proliferation of bovine PBMCs to HEL. Proliferation of freshly isolated bovine PBMC preparations stimulated with either a) 1 μ g/ml HEL or b) 10 μ g/ml HEL were measured by the incorporation of [3H]-thymidine during the course of a 28 day trial in which cows received either 250 μ g HEL (●) or 500 μ g HEL (■).

7.3.2 Humoral Immune responses in the presence of purified Shiga toxin 2

To determine the effects of Stx2 on bovine immune responses to HEL during active immunization, a trial was designed involving 16 Holstein cows divided into two groups. The control group was immunized twice with HEL alone and the treatment group was immunized with HEL and purified Stx2. The results (Figure 22) indicated that Stx2 downregulated total IgG antibody activity to HEL. Throughout the duration of the trial, the antibody titre to HEL of the control group increased until approximately day 21. On day 21, both groups were boosted and the antibody activity increased again in the control group. The antibody activity of the treated group (receiving 500µg HEL and 2µg Stx2) did not follow the same pattern as the control group (receiving 500µg HEL only) remaining significantly lower throughout the 30 days of the trial (Figure 22). To account for the repeated measures design of this experiment and the non-normal distribution of the data, differences between the experimental groups, over time, were examined by first summing the data for each animal over time, ranking the data to transform it to an approximate normal distribution and then performing an ANOVA on the ranks of the summed data. When the ANOVA's were significant ($P < 0.05$) differences between treatments at each time point were tested using the Wilcoxon Rank Sum Test. Therefore based on these statistical parameters, data collected on days 10, 14, 21, 23, 28 and 30 were significantly different between the control and treated groups (Table 7).

The levels of two antibody subtypes, IgG1 and IgG2, to HEL were also measured throughout the trial (Figures 23 and 24). The results showed a very similar pattern to the total IgG antibody activity discussed above. Based on the statistical parameters outlined above, IgG1 and IgG2 antibody activity on days 14, 21, 23, 28 and 30 were significantly different between the control and treated groups (Table 7). Therefore the data presented suggests a significant downregulation of bovine humoral immune responses in the presence of purified Stx2 during active immunization.

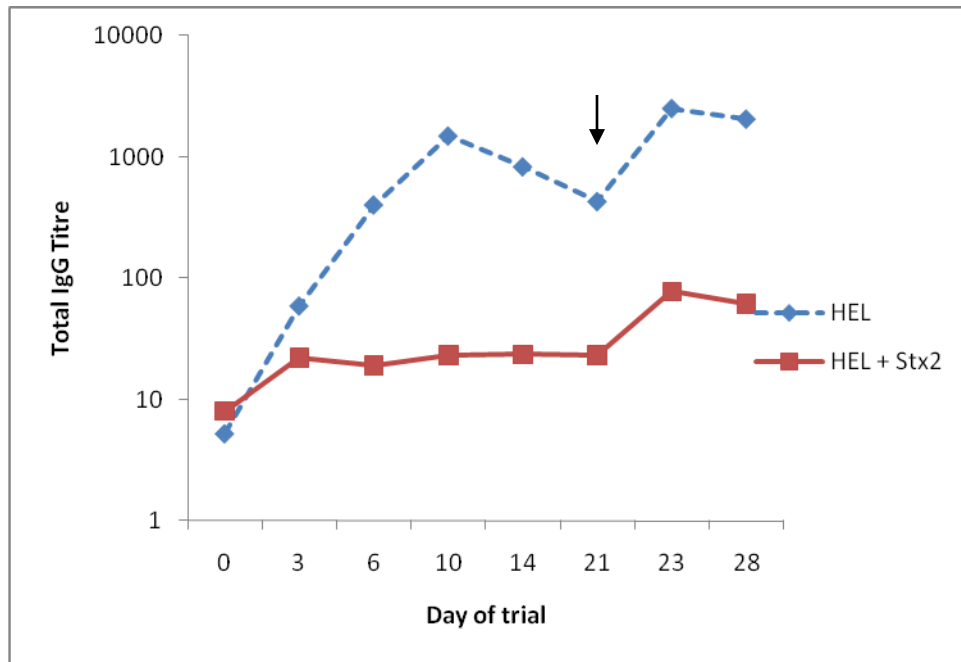


Figure 22. Kinetics of the total HEL-specific IgG antibody production during the course of a 30 day trial. Serological responses to HEL (— —) and HEL + Stx2 (—) were measured using ELISAs as described above. Cows were vaccinated with 500µg of HEL followed by one boost, on day 21 of the trial. The arrow represents vaccinations given at day 21.

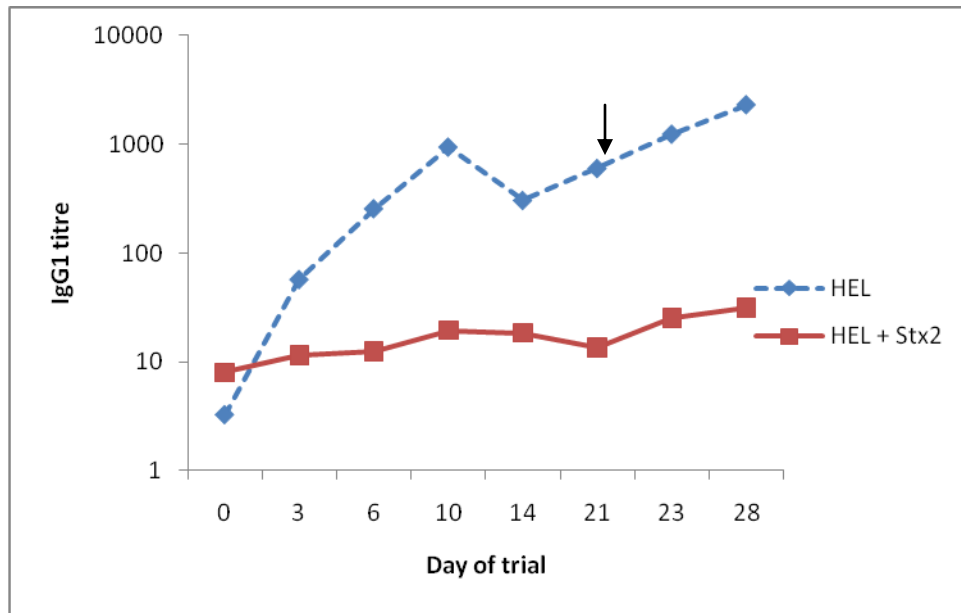


Figure 23. Kinetics of the HEL-specific IgG1 antibody production. Serological responses to HEL (— —) and HEL + Stx2 (—) were measured using ELISAs as described above. Cows were vaccinated with 500µg of HEL followed by one boost, on day 21 of the trial. The arrow represents vaccination given at day 21.

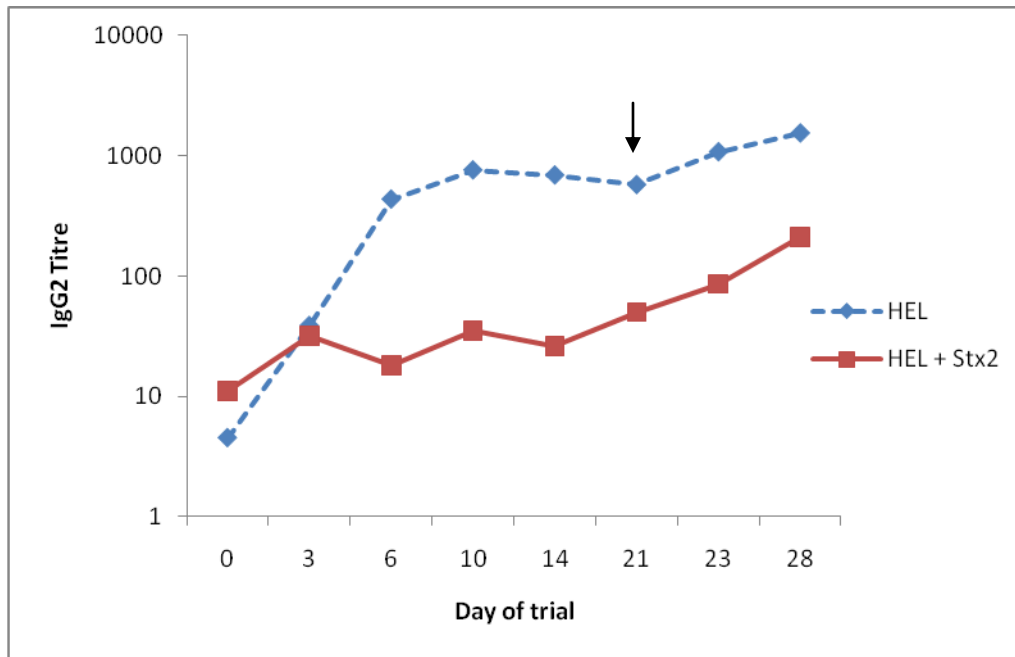


Figure 24. Kinetics of the HEL-specific IgG2 antibody production. Serological responses to HEL (— —) and HEL + Stx2 (—) were measured using ELISAs as described above. Cows were vaccinated with 500µg of HEL followed by one boost, on day 21 of the trial. The arrow represents vaccination given at day 21.

	Day 3	Day 6	Day 10	Day 14	Day 21	Day 23	Day 28	Day 30
Total IgG	0.2424	0.3170	0.0054	0.010	0.0009	0.0009	0.0009	0.0009
IgG1	0.1394	0.1275	0.0180	0.0054	0.0011	0.0009	0.0009	0.0009
IgG2	0.2691	0.4945	0.0831	0.0239	0.0180	0.0927	0.010	0.0136
Proliferation	-	-	-	0.0078	0.0023	-	0.0114	-

Table 7. Statistical analysis of the serological responses (total IgG, IgG1 and IgG2) and proliferative responses to HEL. To account for the repeated measures design of this experiment and the non-normal distribution of the data, differences between the experimental groups, over time were examined by first summing the data for each animal over time, ranking the data to transform it to an approximate normal distribution and then performing an ANOVA on the ranks of the summed data. When the ANOVA's were significant ($P < 0.05$) differences between treatments at each time point were tested using the Wilcoxon Rank Sum Test. Based on these statistical parameters, data collected on days 10, 14, 21, 23, 28 and 30 were significantly different (in bold) between the control and treated groups.

7.3.3 Cellular Immune responses in the presence of purified Shiga toxin 2

To determine the effects of Stx2 on bovine cellular immune responses during active immunization, a trial was designed involving 16 Holstein cows divided into two groups. The control group was immunized twice with HEL alone and the treatment group was immunized with HEL and purified Stx2. PBMCs were isolated from blood samples and bovine proliferative responses to HEL were determined using the incorporation of [³H]-thymidine. The results (Figure 25) showed that bovine proliferative responses were downregulated in the presence of Stx2. On day 14, a stimulation index (SI) value for the treated group was approximately 1.0 and the SI value for the control group was approximately 3.5. On day 21, an SI value for the treated group was approximately 0.7 and the SI value for the control group was approximately 2.0. On day 28, an SI value for the treated group was approximately 1.0 and the SI value for the control group was approximately 3.9 (Figure 25). Statistical analysis using the Mann-Whitney test indicated that cows immunized in the presence of Stx2 (treated group receiving 500µg HEL and 2µg Stx2) had significantly lower proliferative responses on days 14, 21 and 28 upon restimulation with 10µg/ml HEL than cows immunized in the absence of Stx2 (control group receiving 500µg HEL) (Table 7).

7.3.4 Effect of Shiga toxin 2 on Cytokine Production

To determine the effects of Stx2 on bovine cytokine production during active immunization, IFN γ and IL4 concentrations were determined using the same group of cows described above. The number of IFN γ producing PBMCs was determined numerically upon restimulation with 10µg/ml HEL on days 14, 21 and 28 of the trial. Upon comparing the data collected from the treated group with the control group, it was established that there was no measurable difference between the two groups on any of the days tested (Figure 26). IL4 cytokine concentrations were also evaluated on days 14, 21 and 28 of the trial. The results showed that IL4 concentrations did not differ significantly between the two groups on any of the days tested (Figure 27). These results suggest that Stx2 does not modulate antibody or proliferative responses by perturbing IFN γ or IL4 concentrations and the downregulation in antibody and proliferative responses observed were the result of a different mechanism.

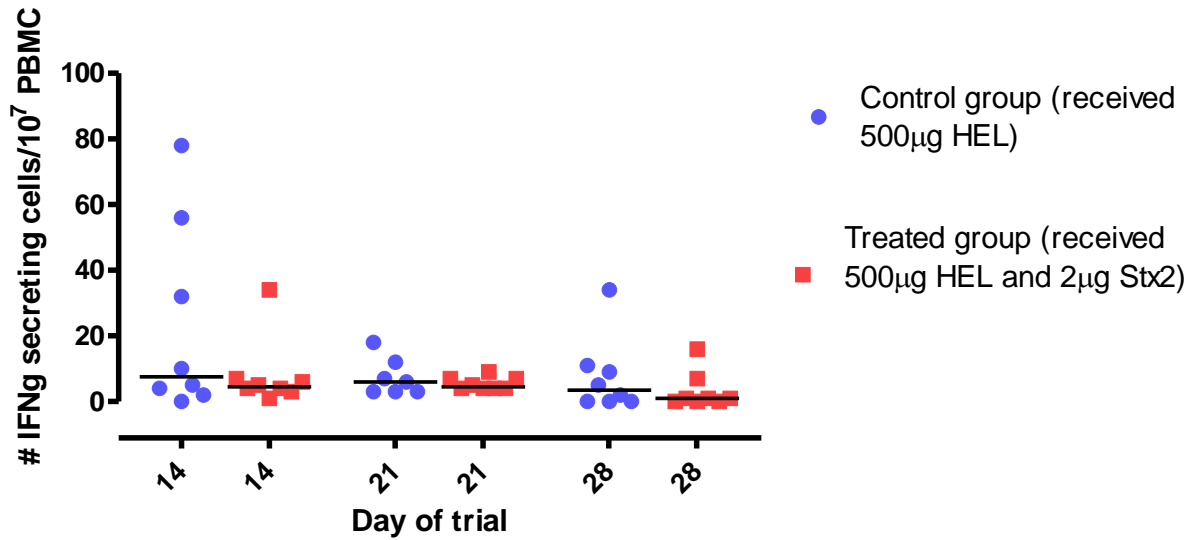


Figure 26. IFN γ production by bovine PBMCs in the presence of Stx2 compared to IFN γ production in the absence of Stx2. Freshly isolated bovine PBMCs from cows vaccinated with either HEL (●) or HEL + Stx2 (■) were restimulated with 10 μ g/ml HEL for 96 hours at 37°C under 5% CO₂ and the resulting number of IFN γ secreting cells numerically counted as described above. Statistical analysis using the Mann-Whitney test indicated that IFN γ production did not differ significantly ($P < 0.05$) on days 14, 21 and 28 upon comparison of the control group and the treated groups.

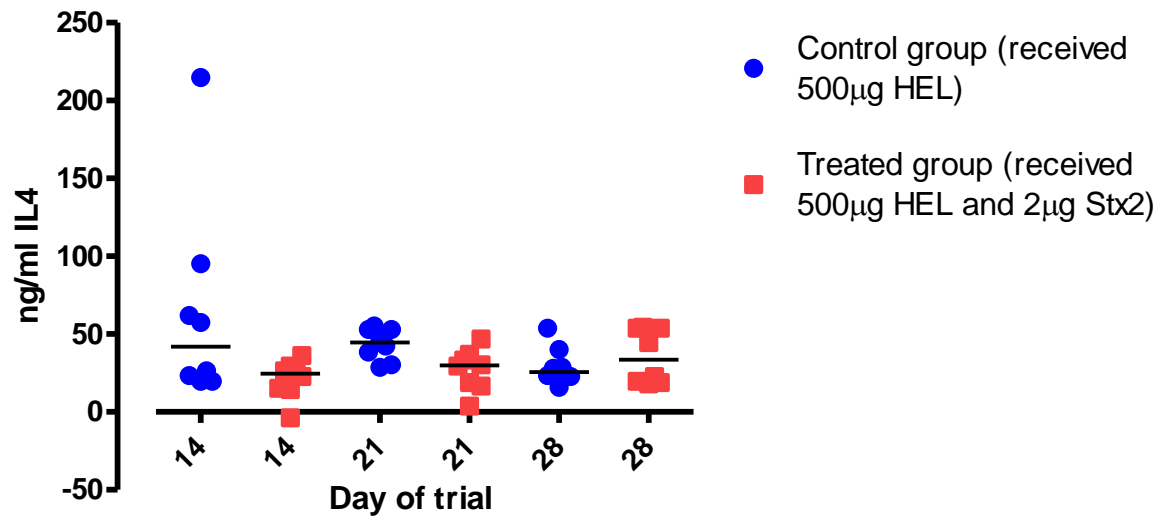


Figure 27. IL4 production by bovine PBMCs in the presence of Stx2 compared to IL4 production in the absence of Stx2. IL4 concentrations determined from cows vaccinated with either HEL (●) or HEL + Stx2 (■) during the course of a 30 day trial. Statistical analysis the Mann-Whitney test indicated that IL4 production did not differ significantly ($P < 0.05$) on days 14, 21 and 28 upon comparison of the control group and the treated groups.

7.3.5 Characterization of Secreted protein preparations to Determine the Effect of Stx2 on Bovine Immune Responses

Vaccination with the current secreted protein vaccine reduces the number of *E. coli* O157:H7 shed in feces, the numbers of animals that shed *E. coli* O157:H7, and the duration of shedding in an experimental challenge model [162]. The secreted proteins used for immunization in the trial were isolated from an Stx2⁺ *E. coli* O157:H7 strain. The objective of the current study was to determine if Stx2 downregulates bovine immune responses during active immunization, therefore immunization of cattle with secreted protein preparations isolated from a wild type Stx2⁺ *E. coli* O157:H7 strain and an isogenic mutant Stx2⁻ *E. coli* O157:H7 strain was carried out. The secreted proteins were isolated from both *E. coli* strains and western analysis was performed on both protein preparations to confirm the presence of secreted proteins (Figure 28). A polyclonal antibody preparation to *E. coli* O157:H7 secreted proteins was produced in our lab by D. Asper and used in the present study. Figure 28 confirmed the presence of secreted proteins in both protein preparations. The concentrations of both protein preparations were also determined and we showed that both secreted protein preparations contained 1µg/µl of secreted proteins (Table 8).

Western blot analysis was done after PAGE separation to confirm the presence of Stx2 in the secreted protein preparation isolated from the wildtype Stx2⁺ *E. coli* strain and the absence of Stx2 in the secreted protein preparation isolated from the mutant Stx2⁻ *E. coli* strain. A monoclonal antibody to Stx2 confirmed Stx2 expression and production from the wild type *E. coli* O157:H7 strain and also confirmed the absence of Stx2 production from the mutant *E. coli* O157:H7 strain. By comparison with the low molecular weight protein ladder used during the western blot analysis we showed a single band migrating in the 60kDa protein range (Figure 29). The molecular weight of Stx2 is approximately 65 kDa. The cytotoxic activity of the secreted protein preparations was determined as described above (Section 7.2.9) using VERO cells as the target. The results of the assays (Figure 30) showed that the secreted proteins isolated from the wild type Stx2⁺ *E. coli* O157:H7 strain had a CD₅₀ value of 100pg/ml and the preparation isolated from the mutant Stx2⁻ *E. coli* strain had an approximate CD₅₀ value of >100ng/ml, indicating that the total secreted protein preparation isolated from the mutant Stx2⁻ strain lacked cytotoxic activity.

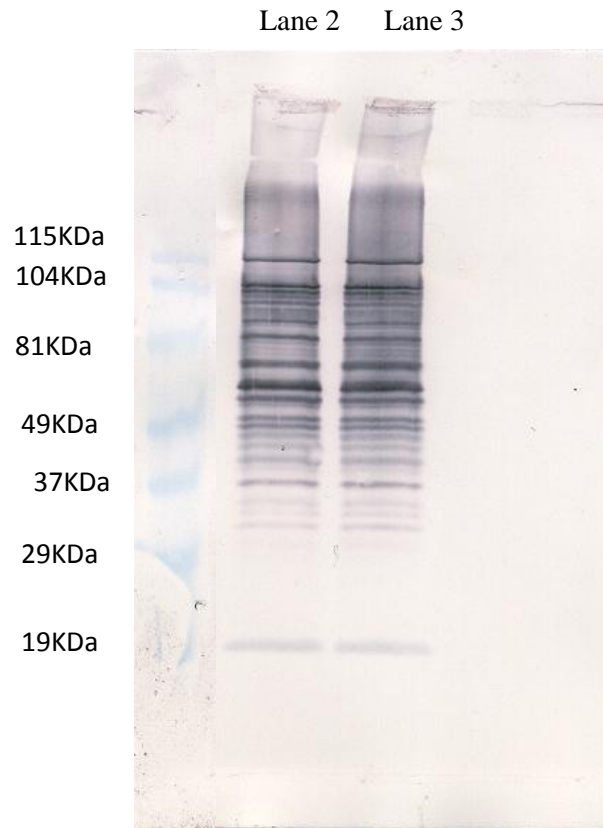


Figure 28. Western blot analysis of the secreted proteins. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed with a polyclonal antibody to secreted proteins produced at VIDO by D. Asper. Lane 2 = secreted proteins isolated from the wild type Stx2⁺ *E. coli* O157:H7 strain and lane 3 = secreted proteins isolated from the mutant Stx2⁻ *E. coli* strain.

Volume	OD ₇₅₀ wild type preparation	OD ₇₅₀ mutant preparation
10ul	0.019	0.018
20ul	0.030	0.024
40ul	0.045	0.041

Table 8. The concentrations of the secreted protein preparations determined using a standard BSA graph. The OD₇₅₀ of three different amounts of protein (10μl, 20μl and 40μl) were measured as described above. The OD readings were used to extrapolate the protein concentrations from the BSA standard curve. It was calculated that both protein preparations were 1μg/μl.

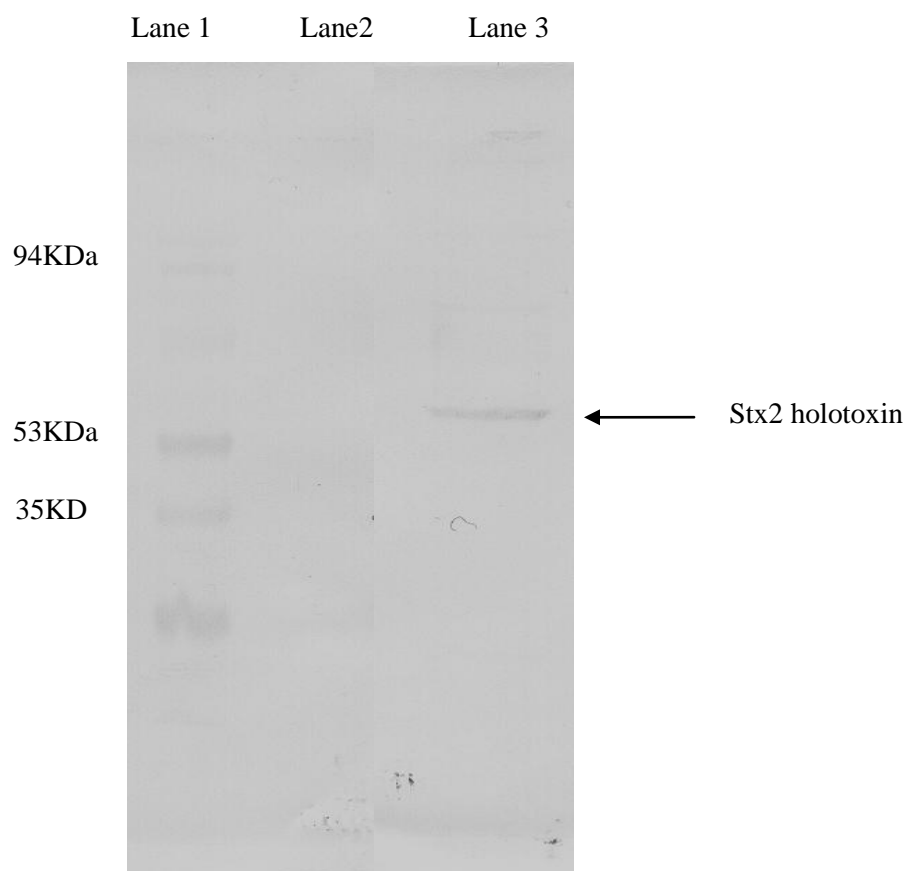
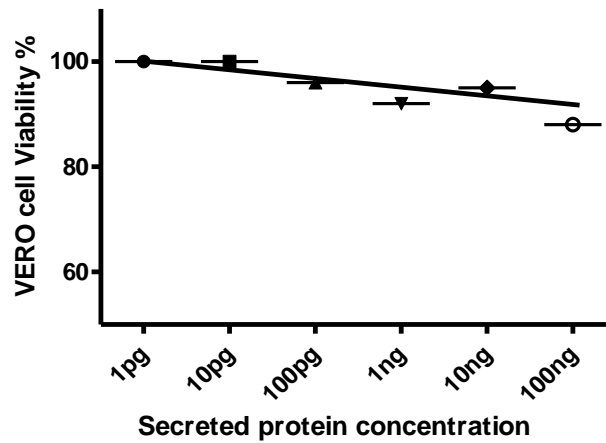


Figure 29. Western blot analysis of secreted proteins. Lane 2 = the mutant Stx2⁻ *E. coli* O157:H7 strain (92192) secreted protein preparation and lane 3 = the wild type Stx2⁺ *E. coli* O157:H7 strain (1961) secreted protein preparation. These results demonstrate that the protein recognized by the Stx2 monoclonal antibody is present in the secreted protein preparation isolated from the wild type Stx2⁺ *E. coli* strain.

a.

Cytotoxicity of STEC Secreted Proteins Isolated from Stx2⁻ Mutant Strain



b.

Cytotoxicity of STEC Secreted Proteins Isolated from Stx2⁺ Wildtype Strain

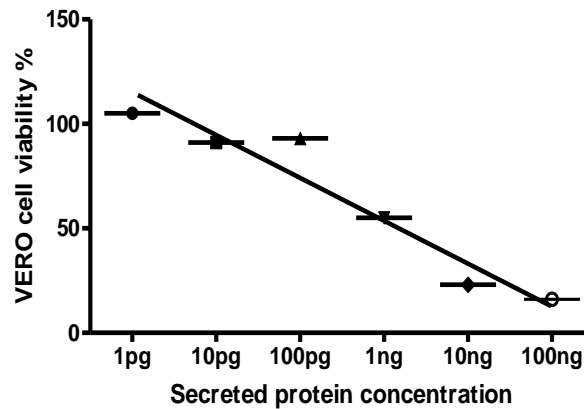


Figure 30. Cytotoxicity of STEC secreted proteins as determined by MTT reduction. The secreted protein preparations were isolated from the a) mutant Stx2⁻ *E. coli* O157:H7 strain (92192) and the b) wild type Stx2⁺ *E. coli* O157:H7 strain (1961). Both secreted protein preparations were diluted in a 10-fold dilution series and incubated with VERO cells (1×10^6) for 96 hours at 37°C.

7.3.6 Humoral Immune responses in the presence of secreted proteins

To determine the effects of Stx2 on bovine immune responses during active immunization, a trial was designed involving 15 Holstein cows divided into three groups (containing 5 cows per group). Group 1 was immunized with secreted proteins isolated from a wild type Stx2⁺ *E. coli* O157:H7 strain (1961) and HEL. Group 2 was immunized with a protein preparation isolated from a mutant Stx2⁻ *E. coli* strain (92192) and HEL and group 3 was immunized with a protein preparation isolated from a mutant Stx2⁻ *E. coli* strain (92192), purified Stx2 and HEL. Serum samples were collected and immune responses against HEL and secreted proteins were determined (Figures 31-33). The results indicated that Stx2 downregulated total IgG, IgG1 and IgG2 antibody activity to HEL. Very little antibody could be detected until day 6 of the trial. From day 6 to day 21, the total IgG titer of group 2 (without Stx2) increased. On day 21, all three groups were boosted and the antibody activity continued to increase in group 2. The antibody activity of groups 1 and 3 did not follow the same pattern as group 2 (without Stx2) but remained significantly lower throughout the 30 days of the trial. Statistical analysis revealed that data collected on days 14, 21, 28 and 35 were significantly different between group 2 (without Stx2) and groups 1 and 3 (Tables 9 and 10). No statistical difference was detected between groups 1 and 3 throughout the entire trial. The results also showed that total IgG antibody activity to secreted proteins was downregulated in the presence of Stx2 (Figure 33) and that the antibody activity followed the same trend noted above to HEL.

7.3.7 Cellular Immune responses in the presence of secreted proteins

To determine the effects of Stx2 on bovine immune responses during active immunization, cellular immune responses to HEL were also measured on days 21 and 28 of the trial. Freshly isolated bovine PBMCs were restimulated with 10µg/ml of HEL for 96 hours and proliferation was measured by the incorporation of [³H]-thymidine. The results indicated that on both days, proliferation was slightly higher in cells from the animals immunized with the mutant Stx2⁻ secreted protein preparation (Group 2) relative to the cells from the animals immunized with the Stx2-containing formulations (Groups 1 and 3). Statistical analysis using the Dunn's Multiple Comparison Test following one-way repeated ANOVA indicated no significant difference between the three groups on either day tested. These results differed from the

proliferation results in Trial 2 (Figure 25), when animals were immunized with purified Stx2 alone and proliferation was found to be statistically different between the control and treated groups. In the current trial, proliferation could have been suppressed by other proteins/components of the secreted protein preparations thus creating an overall suppression in all three groups. A general trend suggesting slightly higher proliferation in group 2 was observed (Figure 34) but this not statistically different compared to groups 1 and 3.

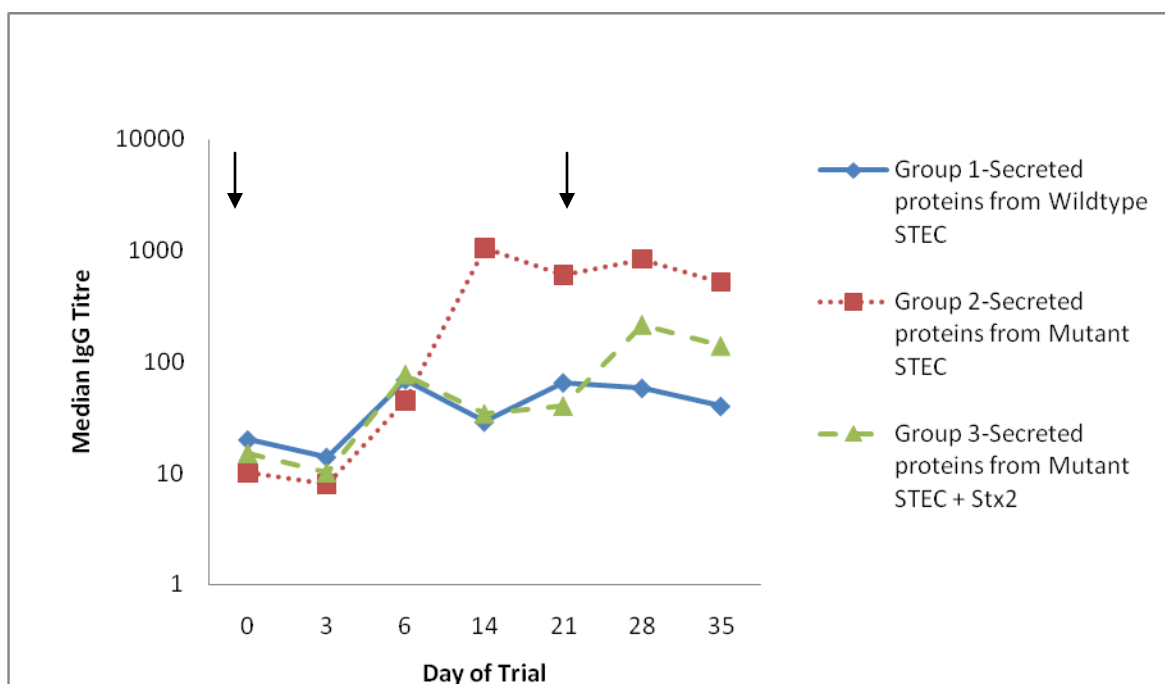


Figure 31. Kinetics of the total HEL-specific IgG antibody during the course of a 35 day trial. Animals were immunized with STEC secreted proteins and HEL in the presence (—, — — —) and absence of Stx2 (.....). Serological responses to HEL were measured using ELISA as described above. Arrows represent vaccinations given at day 0 and day 21. The Dunn's Multiple comparison analysis was done at each time point between all three groups and the results of the statistical analysis are shown in Tables 9 and 10.

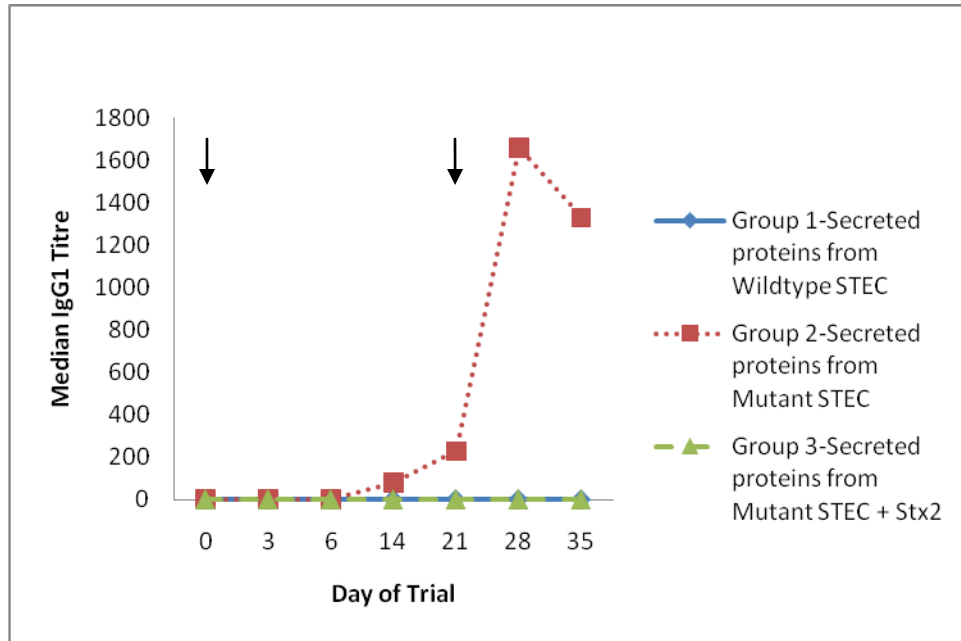


Figure 32. Kinetics of the total HEL-specific IgG1 antibody during the course of a 35 day trial. Animals were immunized with STEC secreted proteins and HEL in the presence (— and — — —) and absence of Stx2 (····): The serological responses to HEL were measured using ELISA as described above. Arrows represent vaccinations given at day 0 and day 21. The results of the statistical analysis are in Tables 9 and 10.

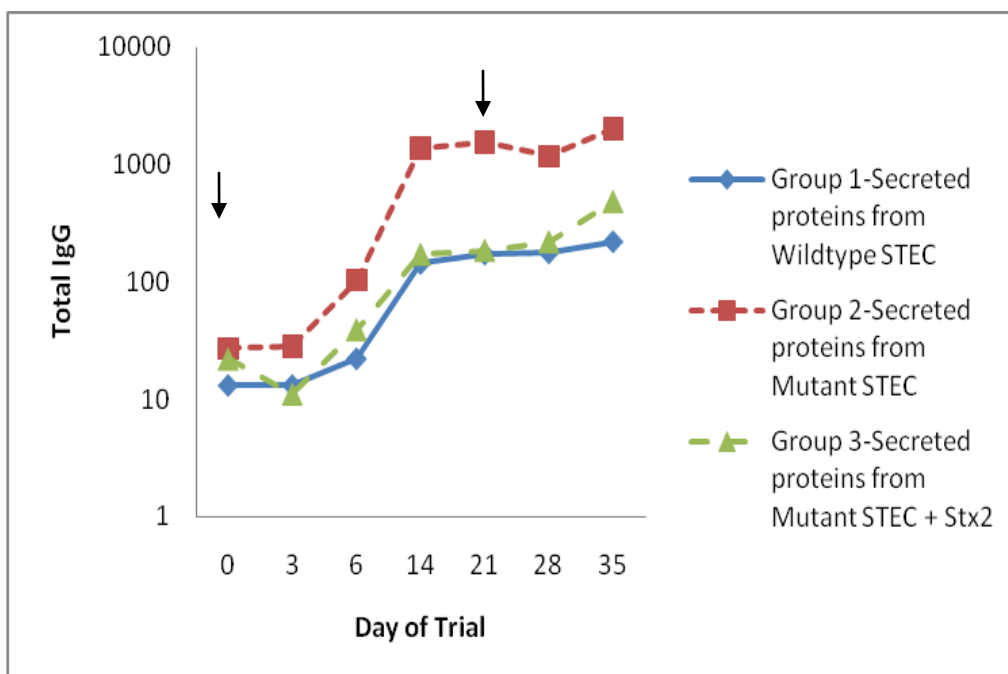


Figure 33. Kinetics of the total secreted protein-specific IgG antibody during the course of a 35 day trial. Animals were immunized with STEC secreted proteins and HEL in the presence (— and - -) and absence of Stx2 (.....). The serological responses to secreted proteins were measured using ELISA as described above. Arrows represent vaccinations given at day 0 and day 21. The results of the statistical analysis are in Tables 9 and 10.

	P Values						
	Day 0	Day3	Day6	Day14	Day21	Day28	Day35
Total HEL-specific IgG	0.2222	0.8340	1.000	0.0080	0.0317	0.0079	0.0159
HEL-specific IgG1	*	*	0.9063	0.1188	0.0200	0.0179	0.0342
Total secreted protein-specific IgG	0.6752	0.6905	0.4020	0.0079	0.0080	0.0079	0.0079

* p Value could not be determined due to lack of detectable titer.

Table 9. P values determined upon comparing serological responses to HEL through the trial. Serological responses from cattle that received the secreted protein preparation isolated from the wild type STEC strain were compared to serological responses determined from cattle that received the secreted protein preparation isolated from the Stx2⁻ mutant STEC supplemented with purified Stx2. Statistical significance has been defined as $P < 0.05$ and is shown in bold. The results indicated that total IgG antibody activity to HEL were significantly different on days 14, 21, 28 and 35 of the trial and IgG1 antibody activity was significantly different on days 21, 28 and 35 of the trial.

	P value						
	Day 0	Day3	Day6	Day14	Day21	Day28	Day35
Total HEL-specific IgG	0.5476	0.8413	0.9166	0.0079	0.0159	0.0079	0.0952
HEL-specific IgG1	*	*	0.6072	0.1612	0.0079	0.0079	0.0079
Total secreted proteins-specific IgG	0.4206	0.5476	0.5476	0.0079	0.0119	0.0079	0.0079

* p Value could not be determined due to lack of detectable titer.

Table 10. P values determined upon comparing serological responses in cattle throughout the trial. Cows that received the protein preparation isolated from the Stx2⁻ strain were compared to the serological responses from the cows that received the secreted protein preparation isolated from the Stx2⁻ strain supplemented with purified Stx2. Statistical significance has been defined as $P < 0.05$ and is shown in bold. The results indicate that total IgG antibody activity to HEL were significantly different of days 14, 21, 28 and 35 of the trial and IgG1 antibody activity was significantly different on days 21, 28 and 35 of the trial.

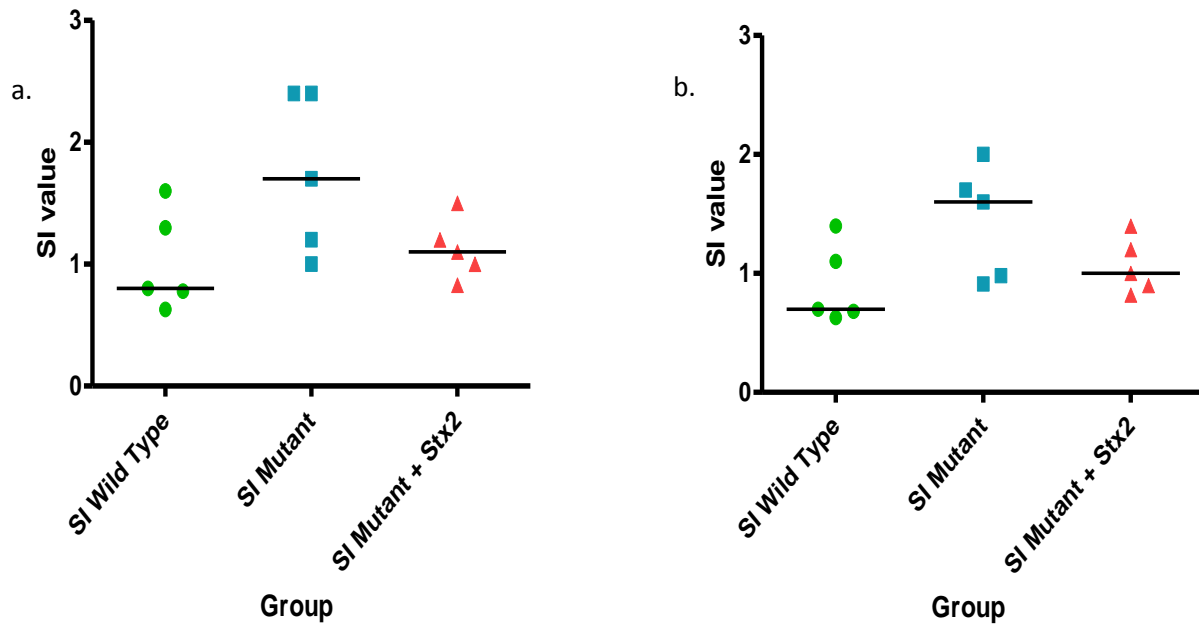


Figure 34. Proliferation of bovine PBMCs in the presence of Stx2. Bovine PBMCs were isolated from cows immunized with either secreted proteins isolated from the wild type STEC strain (●), secreted proteins isolated from the Stx2⁻ mutant strain (■) or secreted proteins from the Stx2⁻ mutant strain supplemented with purified stx2 (▲) and proliferation was measured on a) day 21 and b) day 28 using ³H-thymidine as described above.

7.4 Discussion

Several vaccine trials using secreted proteins have been attempted in order to reduce the prevalence of STEC in cattle and therefore reduce the incidence of human exposure to STEC. In 2004 Potter et al [162] successfully reduced colonization of STEC by vaccinating with several type III secretion proteins. The results showed that vaccination resulted in a significant reduction in the numbers of bacteria shed in feces, the numbers of animals that shed, and the duration of shedding in an experimental challenge model [162]. One year later, Peterson et al [230] conducted a feedlot trial using secreted proteins (isolated using the same protocol as Potter et al [162]) from a wild type *E. coli* O157:H7 strain and showed that vaccination reduced the number of cattle shedding *E. coli* O157:H7. Finally in 2009, Smith et al [169] showed that 2.9% of the terminal rectal mucosa of cattle vaccinated with the secreted protein preparation isolated from wild type *E. coli* O157:H7 were colonized and 17% of the terminal rectal mucosa were colonized in non-vaccinated animals. Therefore published data exists to support the hypothesis that vaccination with secreted proteins does reduce *E. coli* O157:H7 carriage in cattle. Even though published data exists supporting vaccination in cattle as a means to reduce STEC, commercially available vaccines are not being used by farms and STEC remain a significant zoonotic pathogen of humans causing disease and death. In the present study, we hypothesized that immunity to STEC may not be optional due to Stx2 and thus determined the effect of Stx2 on the bovine immune response during active immunization.

The Stxs are a major STEC virulence factor and have been implicated in *E. coli* O157:H7 disease pathogenesis in humans [3] and immunomodulation in cattle [190]. There is a growing amount of data to suggest that the Stxs in cattle leads to immune suppression and persistent STEC colonization. Downregulation of bovine immune responses by Stx2 (acquired from STEC infection or from vaccination) may therefore make immunity to STEC and other co-administered vaccines not possible. Menge et al showed that Stx1 specifically targets bovine CD8⁺ lymphocytes within the ileum of calves [203]. They demonstrated a significant reduction in the percentage of CD8⁺ intraepithelial lymphocytes after 12 hours of incubation with a Stx1⁺ *E. coli* strain. *In vitro* studies have shown that Stx1 is also able to inhibit bovine peripheral lymphocyte proliferation suggesting a general immune suppression [68]. Also it has been reported that calves inoculated with Stx2⁺ *E. coli* O157:H7 have significantly higher and longer

fecal shedding of *E. coli* O157:H7 compared to calves inoculated with Stx2⁻ *E. coli* O157:H7 or a non-pathogenic *E. coli* [190]. We have data suggesting that Stx2 inhibits bovine lymphocyte proliferation *in vitro* as well as reduces the number of CD4⁺ T cells *in vivo* during a bovine ligated loop model of STEC infection. To extend this, two trials were performed in which cattle were immunized either in the presence or absence of Stx2 and the magnitude of bovine immunological responses (humoral and cellular) was measured. The data indicates that Stx2 alone as well as part of the secreted proteins isolated from a wild type Stx2⁺ *E. coli* O157:H7 strain reduced bovine immune responses to an academic antigen HEL as well as the secreted proteins themselves. These results suggest that vaccination with a secreted protein preparation that does not contain Stx2 (secreted protein preparation isolated from a mutant Stx2⁻ *E. coli* strain) could result in significantly better immunological responses in cattle compared to vaccination with a secreted protein preparation that does contain Stx2.

The data in the present study suggests that Stx2 downregulates bovine immune responses during active immunization. Stx2 is likely required for long-term colonization of the bovine host and this is supported by the observation that Stx2⁺ *E. coli* O157:H7 were able to colonize cattle for longer periods of time compared to Stx2⁻ *E. coli* O157:H7 [190]. This would imply that Stx2⁺ *E. coli* O157:H7 remain in the gastrointestinal tract for longer periods of time enabling them to produce sufficient biologically active amounts of Stx2 within the gut lumen. This free Stx2 would be available to interact with receptor-bearing cells. Menge et al [203] showed that bovine lymphocytes isolated from the gastrointestinal tract as well as the blood express surface receptors for Stx2. We have data confirming the presence of surface Gb₃ on bovine lymphocytes isolated from the Peyer's patches of the ileum and jejunum. It was demonstrated by Stamm et al [178] that a certain percentage of Gb₃-positive lymphocytes bound Stx2 but did not internalize the toxin suggesting that Stx2 may perturb normal cell functions through this interaction. Therefore, we propose the potential mechanism by which Stx2 might downregulate bovine immune responses is by remaining bound to Gb₃⁺ lymphocytes within the gastrointestinal tract thereby inhibiting lymphocyte proliferation and thus the generation of an immunological response. We have data demonstrating an inhibition of lymphocyte proliferation in the presence of purified Stx2 *in vitro*. We have also shown a reduced number of bovine CD4⁺ T cell in the presence of Stx2 *in vivo* suggesting Stx2 might inhibit proliferation of this subgroup of

lymphocyte. Menge et al [203] showed that Stx1 specifically targets bovine CD8⁺ lymphocytes within the ileum of calves. The results indicated a significant reduction in the percentage of CD8⁺ intraepithelial lymphocytes after 12 hours of incubation with a Stx1⁺ *E. coli* strain. Other toxins have also been shown to have immunomodulatory roles in animal models including the two anthrax toxins called Lethal toxin and Edema toxin [236]. Comer et al [236] showed that injection of mice with sublethal doses of either toxin inhibited the proliferation of T cells. The data in the present study demonstrated reduced cellular immune responses in the presence of Stx2 implying an immunomodulatory role for Stx2 in bovines. Hoffman et al [190] showed calves with prior exposure to an *E. coli* O157:H7 (Stx⁺) strain lacked significant lymphocyte proliferative responses to a heat-killed *E. coli* O157:H7 Stx2⁺ strain suggesting infections with STEC can suppress immune responses in cattle. In addition, Christopher-Hennings et al [237] showed that gnotobiotic pigs persistently infected with a STEC strain had significantly reduced antibody titres to sheep erythrocytes as well as lowered proliferative responses to subsequent exposure to mitogens such as ConA suggesting persistent infection is associated with a downregulation in immunological responses. Therefore, based on these findings as well as the current study, a requirement for a Stx2⁻ *E. coli* mutant protein preparation when immunizing cattle with a secreted protein cocktail should be taken into consideration for future vaccination.

8.0 Discussion and Conclusions

STEC are a cause of severe illness and death in humans, and because cattle are an important reservoir for human exposure, it is important to reduce the amount of STEC in cattle to reduce human exposure and thus disease [238]. To date, very little is known about STEC pathogenesis in cattle in particular the virulence factors associated with long-term persistence. It has been shown that cattle shed STEC for long periods of time throughout their lifespan without causing any clinical symptoms of disease [1, 169, 198]. Bacterial pathogens use a variety of mechanisms including toxins, to avoid immune responses and successfully colonize their hosts [46]. This has been reported for STEC but the mechanism has yet to be defined. Therefore the primary objective of this project was to determine the effect of Shiga toxin 2 on bovine immune responses during STEC infection. We hypothesized that STEC uses Shiga toxin 2 to downregulate bovine immune responses thereby preventing the induction of the immune system and subsequent elimination from its host.

In this project, we hypothesized that Stx receptor-bearing lymphocytes are susceptible to Stx2 thereby altering normal lymphocyte functions. Our results showed that leukocyte populations isolated from the jejunum and the ileum of cattle as well as an intestinal epithelial cell line express the Stx (Gb₃) receptor on their surface. Dean-Nystrom et al [70] showed that the ileum and colon of these experimentally infected calves were colonized with viable bacteria thus confirming the presence and tissue tropism of STEC within the bovine gastrointestinal tract. In accordance with this, Menge et al [203] showed that bovine lymphocytes isolated from the gastrointestinal tract as well as the blood express surface receptors for Stx2. Stx receptor binding has been shown to be the principle determining factor in Stxs specificity and activity as demonstrated in pigs and rabbits [58]. Therefore, Stx receptor-bearing cells within the gastrointestinal tract would be potential targets during STEC colonization. Results in the current study have shown that Stx2 inhibits the mitogen-induced proliferation of lymphocytes originating from the ileal Peyer's patches. This inhibition was seen after 12 hours of incubation with purified Stx2 (2000pg/ml) and 5µg/ml of Concavallin A. *In vitro* experiments have also demonstrated reduced lymphocyte proliferation in the presence of purified Stx1 [68]. To further these results, an *in vivo* analysis using a ligated loop model with a wild type Stx2⁺ *E. coli* O157:H7 strain demonstrated the depletion of bovine CD4⁺ T cells within the ileum suggesting

part of the mechanism by which Stx2 facilitates STEC colonization is through the modulation of the bovine immune response. Menge et al [203] showed that Stx1 specifically depletes bovine CD8⁺ lymphocytes within the ileum of calves and reduces the percentage of CD8⁺ intraepithelial lymphocytes after 12 hours of incubation with an Stx1⁺ *E. coli* strain. The present study provides direct evidence that Stx2 produced during STEC infection can modulate lymphocyte populations in the mucosal immune system within cattle.

The epithelial cells lining the mucosal surface of the gastrointestinal tract are often the host's first line of defence providing a physical barrier between STEC and the host's internal environment [207]. In the gastrointestinal tract, the epithelial cells play an active role in producing signals that direct the neighbouring immune and inflammatory cells in the mucosa [207]. Therefore in this project, we hypothesized that Stx2 downregulates cytokine gene expression from the bovine intestinal epithelium thereby preventing the induction of important inflammatory cytokines. *In vitro* analysis of the effect of Stx2 on cytokine gene expression at the transcriptional level in a bovine intestinal epithelial cell line showed that cytokine induction was mildly suppressed in the presence of Stx2. Data resulting from a purified Stx2 preparation and a secreted protein preparation isolated from a wild type Stx2⁺ *E. coli* O157:H7 strain showed a slight downregulation of IL12, IL6, IL8 and IFN γ gene expression compared to cytokine gene expression in the absence of Stx2. These results could suggest that Stx2 has a role in STEC colonization since there are no signs of intestinal inflammation in STEC-infected cattle [219]. In agreement with our results, Bellmeyer et al [217] recently showed that *E. coli* O157:H7 attenuates the induction of IL8 as well as inflammatory responses by intestinal epithelial cells. As well, Hauf et al [216] confirmed that EHEC suppresses NF- κ B activation thereby inhibiting inflammatory processes. Therefore, our results in conjunction with published data, suggests Stx2 impairs bovine inflammatory processes by downregulating cytokine gene expression and is thus part of the pathogenic mechanism of STEC colonization.

With the growing amount of published data confirming the immunomodulation of Stx2 within the bovine host [68, 69, 219], it is imperative to determine the effect of Stx2 during active immunization. Therefore in the present study, two trials were performed in which cattle were immunized either in the presence or absence of Stx2 and the magnitude of bovine immunological responses (humoral and cellular) were measured. Our results indicates that Stx2 alone as well as

part of the secreted protein preparation isolated from a wild type Stx2⁺ *E. coli* O157:H7 strain reduced bovine immune responses to a co-administered antigen, HEL, and to the secreted proteins themselves. These results suggest that vaccination with a secreted protein preparation that does not contain Stx2 (secreted protein preparation isolated from a mutant Stx2⁻ *E. coli* strain) will result in significantly better immunological responses in cattle compared to vaccination with a secreted protein preparation that does contain Stx2.

The data in the present study suggests that Stx2 downregulates bovine immune responses. We hypothesized that STEC uses Shiga toxin 2 to downregulate bovine immune responses thereby preventing the induction of the immune system and subsequent elimination from its host. Stx2 is likely required for long-term colonization of the bovine host and this is supported by the observation that Stx2⁺ *E. coli* O157:H7 were able to colonize cattle for longer periods of time compared to Stx2⁻ *E. coli* O157:H7. This would imply that Stx2⁺ *E. coli* O157:H7 remain in the gastrointestinal tract for longer periods of time than Stx2⁻ *E. coli* O157:H7 enabling them to produce sufficient amounts of Stx2 within the gut lumen. It is been suggested that a proportion of free Stx2 traverses the intestinal epithelial cell layer [58] and reaches the bloodstream where it travels to distal organs. This has never been substantiated by the finding of free Stx2 within the bloodstream of either cattle or humans. As well, in 1995 Cray et al [188] concluded from their study using preweaned calves and adult cattle that *E. coli* O157:H7 does not spread from the alimentary tract to other organs such as the kidneys, liver or spleen.

Therefore, it is assumed that the majority of free Stx2 is either taken up by specialized M cells situated in the intestinal epithelium and/or traverses the epithelium [58] but remains in the underlying gastrointestinal tract tissues. Here Stx2 is exposed to the gut-associated lymphoid tissue (GALT). Receptors for Stx2 have been identified on bovine lymphocytes located peripherally in the blood as well as in the lamina propria [178]. A study done by Stamm et al [178] showed that a percentage of receptor-bound Stx2 was not internalized but remained bound to surface Gb₃ on lymphocytes. Therefore it is likely that Stx2 interacts with lymphocytes within the GALT without exerting cytotoxic effects [178]. In the present study, we confirm the presence of surface Gb₃ on leukocyte populations (CD45⁺) isolated from the Peyer's patches of the ileum and jejunum of cattle. We speculate that Gb₃⁺ leukocyte populations including lymphocytes (B and T cells), antigen presenting cells and dendritic cells situated in the

gastrointestinal tract bind free Stx2 [178] and that a percentage of receptor-bound Stx2 is not internalized but rather remains bound to surface Gb₃ [178]. Receptor-bound Stx2 possibly perturbs normal cell functions such as proliferation [68]. It has been confirmed that Stx1 hinders bovine lymphocyte proliferation by blocking lymphocyte activation [68]. In the present study we demonstrated an inhibition of mitogen-induced proliferation in lymphocytes isolated from the gastrointestinal tract in the presence of purified Stx2. Therefore we assume that reduced lymphocyte proliferation will affect the magnitude of the immunological response generated during STEC colonization thereby aiding in STEC pathogenesis.

To determine if STEC suppression of bovine mucosal immune responses is important *in vivo*, a bovine ligated loop model was employed. Our results showed a reduction in CD4⁺ T lymphocytes isolated from the Peyer's patches in the presence of a wild type Stx2⁺ strain. This result suggests that Stx2 may preferentially target CD4⁺/Gb₃⁺ T cells within the gastrointestinal tract. Menge et al [203] showed a reduction in CD8⁺ T cells in the presence of a Stx1-producing strain suggesting a possible role for Stx1 in STEC persistence in calves. Therefore, our results support the conclusion that Stx2 suppresses bovine mucosal immune responses. We speculate that the reduced number of lymphocytes within the gastrointestinal tract in the presence of Stx2 is in part due to an inhibition of lymphocyte proliferation as observed in our *in vitro* studies.

In order to address the lack of intestinal inflammation in cattle upon STEC colonization [178], the effect of Stx2 on cytokine production from an intestinal epithelial cell line was defined in the present study. First, confirmation of surface Gb₃ on a bovine intestinal epithelial cell line established by Kaushik et al [204] was performed. Our results showed a reduction in cytokine gene expression from the bovine intestinal epithelial cell line in the presence of Stx2 suggesting Stx2 dampens inflammatory responses during STEC infection. Recently Bellmeyer et al [217] showed that *E. coli* O157:H7 attenuates the induction of IL8 as well as host inflammatory responses by intestinal epithelial cells. Therefore we assume that Stx2 facilitates STEC intestinal colonization by inhibiting inflammatory responses mediated by cytokine production.

Taking into consideration the data in the present study to support the immunomodulatory role of Stx2 during STEC colonization, the question of whether immunity in cattle to STEC and other co-administered antigens was addressed. Our results of two immunization trials in the presence of purified Stx2 and secreted protein preparations containing Stx2 demonstrated

reduced humoral and cellular immune responses in cattle. Johnson et al [102] have confirmed reduced antibody responses to STEC antigens in cattle during experimental infections and Hoffman et al [190] have reported lower lymphocyte proliferation after infection of calves with STEC strains. We therefore do not doubt that an immune response is generated during STEC infection by bacterial components such as LPS and flagellin [70, 216, 218, 239], but we do propose that the magnitude of the immunological response is significantly decreased to STEC proteins as well as other co-administered antigens by Stx2.

Our hypothesis that “STEC uses Shiga toxin 2 to downregulate bovine immune responses thereby preventing the induction of the immune system which contributes to long-term persistence in cattle” was partially proven in the present body of data. We have successfully shown using multiple experiments that bovine immune responses are downregulated in the presence of Stx2 but we have not addressed if this immunological suppression facilitates long-term persistence in the bovine host. In order to further confirm our hypothesis that Stx2 facilitates long-term STEC persistence in cattle, future cattle trials that include STEC challenge after vaccination need to be pursued. Based on the present data, we would predict that cattle vaccinated with a secreted protein cocktail produced from a Stx2⁻ STEC strain would result in reduced colonization compared to cattle vaccinated with a secreted protein cocktail produced from a wildtype Stx2⁺ STEC strain. A decrease in the number of cattle shedding STEC after vaccination as well as a decreased amount of STEC shed in the feces of cattle would suggest a reduction in colonization. Therefore, future experiments are required to confirm our hypothesis regarding the role of Stx2 and STEC persistence in cattle.

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